PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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OBSERVATIONS AND EXPERIMENTS ON DEVELOPMENTAL PATTERN IN PELMATOHYDRA OLIGACTIS[1]

(Twenty-nine figures)

O. RULON AND C. M. CHILD
Hull Zoölogical Laboratory, University of Chicago

EXPERIMENTS dealing with regulation and regeneration in hydra date back to the classical work of Trembley (1744), whose researches have served as a starting-point for numerous other investigations. The great volume of literature that has accumulated on the subject has been adequately reviewed by such workers as Peebles (1897), Koelitz (1910), Hyman (1928), and Weimer (1928), and therefore will not be considered here except as it applies directly to the data gathered in this particular investigation. Numerous results have been obtained, and facts recorded, which we believe lack an adequate interpretation in the light of more recent discoveries. This work therefore serves in part as an attempt at the interpretation and correlation of certain facts which heretofore have been ignored, or at best only partially considered.

The budding phenomenon in hydra is of considerable interest to the developmental physiologist, for it is in the primordia of the bud that the primary developmental patterns first come into being. Questions concerning the budding process at once present themselves. How do the tissues which make up the bud become isolated from the controlling action of the parent? How do polarity and other developmental patterns arise in the bud? How soon, if ever, does a bud reach a stage when the different regions are determined in the formation of such parts as hypostome, body, and stalk? What are the relations between the actively growing bud and the parent?

An attempt has been made in this investigation to answer, at least in part, some of the foregoing questions, although they may never be answered fully without entirely different methods. We have, in this work, sought to throw some light on the origin of the tentacular pattern of the bud. We have endeavored to show something as regards the early determination of the bud. We have striven to point out certain effects of the actively growing bud on the reconstituting parent, as well as the effects of the reconstituting parent on the actively growing bud.

[1] This investigation was supported in part by funds from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago.

MATERIAL AND METHODS

The common brown hydra, *Pelmatohydra oligactis*, was used in this investigation. In large, well-fed individuals of this species the body proper is rather sharply marked off from a more slender, almost transparent stalk about equal in length to the body. The budding region is normally immediately distal to the stalk. The usual number of tentacles is six, although animals are frequently observed with seven, eight, or even more tentacles. The tentacles, when extended, may reach a length several times that of the body.

Because of the phenomenon of "depression" which is common in stocks of hydra (see Hyman, 1928), the well water used in the laboratory cultures was often mixed with water in which hydra had previously lived, and fresh *Elodea* and extracts of boiled wheat were added. With large numbers of hydra, however, it was found that they could themselves condition the well water and survive perfectly well for some weeks without showing the characteristic signs of depression (i.e., shortening of the tentacles, failure to feed, etc.).[2]

Perhaps the greatest difficulty encountered in keeping the animals in the laboratory was the appearance in the cultures of a hypotrichous protozoan, apparently *Kerona*, which parasitized and often killed them. It was found, however, that hydra could be freed of the parasite by washing thoroughly in water at a temperature of 33°–34° C. This procedure usually resulted in loss of tentacles and often in death, but new parasite-free cultures could be started from those hydras which did survive. Apparently, at these temperatures the parasites are less able to adhere to the surface of the host than at lower temperatures.

The animals were fed on *Daphnia*, which are cultured in large numbers in this laboratory. Only rarely was individual feeding, as described by Hyman (1928), attempted. Instead, a large number of *Daphnia* were introduced periodically into the cultures for the hydra to catch and feed upon. Only large animals which had not been fed for 2 or 3 days and which showed well-defined stalks and long tentacles were used in the experiments. Heavily parasitized animals were not used.

In the experiments a number of animals of approximately the same size were removed from the cultures with a medicine dropper to a thin layer of water in a Petri dish. The animals were cut only when fully extended, by a single movement of a small sharp scalpel. Most of the operations, particularly sectioning of buds, were performed under the low power of a dissecting microscope. The sectioned animals were placed in groups in well water in finger bowls and observed at given intervals during reconstitution with the aid of a binocular. All reconstitution took place at room temperature, which ranged from 20° to 25° C. In the controlled experiments the controls and tests were kept under identical conditions.

OBSERVATIONS AND EXPERIMENTS

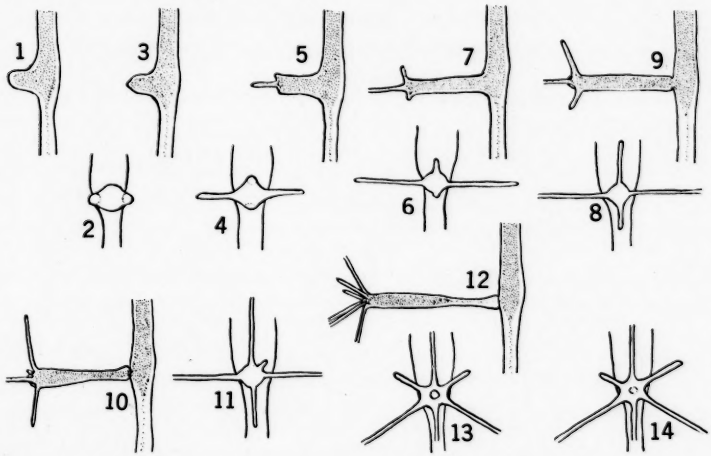
THE PATTERN OF TENTACLE FORMATION IN THE BUD

Perhaps one of the most interesting of recent trends in biological investigation is the determination of pattern in embryological development. We find in the developing bud of hydra a pattern in tentacular appearance which undoubtedly is associated with the

[2] These observations on conditioning are in accord with the work in this laboratory of Professor W. C. Allee and his students, who in recent years have investigated many factors associated with the conditioning process.

axis of the parent animal. Although this pattern in tentacle appearance has been described by various workers (Hertwig, 1906; Koelitz, 1910; Hyman, 1930), we believe it advisable to call attention to it here. Budding in our cultures usually, though not always, was most noticeable at 2–3 days after heavy feeding.

The bud arises as a localized outgrowth in the region immediately distal to the stalk; and as it elongates, it becomes cylindrical with rounded tip, showing in the earlier stages no visible evidence of tentacles (Fig. 1). Soon two slight rounded elevations appear at the lateral margins of the distal tip of the bud in a plane at right angles to the longitudinal axis of the parent (Fig. 2). The gastrovascular cavity of the parent extends into the bud, and at this time (bud between 1 and 2 days old) there is complete continuity between the bud and the parent (Fig. 3).



FIGS. 1–14.—The development of the bud of *Pelmatohydra oligactis*. Figures 1, 3, 5, 7, 9, 10, and 12 show lateral aspect of successive stages in the development of the bud. Figures 2, 4, 6, 8, 11, 13, and 14 show successive stages in the development of tentacular pattern of the bud (Fig. 13 shows the fifth and sixth tentacles arising simultaneously; Fig. 14 at different times in the angles between the first two and third tentacles).

Approximately 24 hours after the first appearance of tentacular buds, tentacle development has reached the stage shown in Figure 4. The body of the bud is slightly more elongated, but there still is complete continuity with the parent (Fig. 5). At this stage the third and fourth tentacles appear at right angles to the first two tentacles in a plane passing through the longitudinal axis of the parent and that of the bud. The third tentacle on the side of the bud toward the apical end of the parent appears before the fourth on the opposite side in the greater number of cases. In fact, the third tentacle is often well advanced before the fourth makes its appearance.

Twelve to 24 hours later the first two tentacles are more elongated, while the third is usually distinctly in advance of the fourth (Figs. 6, 7). When the bud and parent are extended, there seems to be complete continuity between the two; but they may contract separately, and, when contracted, a definite constriction appears at the junction.

Twelve hours later, at room temperature, the bud is longer and the third tentacle is usually well in advance of the fourth (Figs. 8, 9). At this stage a sharp constriction has appeared in the ectoderm at the base of the bud (Fig. 9), but endoderm and gastrovascular cavity of parent and bud are still continuous.

The fifth and sixth tentacles now make their appearance within a very few hours. They arise at the same or different times, but in either event they almost always arise in the angles between the first two and third tentacles (Figs. 10, 11). By this time the bud is separated from the parent by a deep constriction; yet the endoderm is still continuous with that of the parent (Fig. 10). The last stage in budding here described is shown in Figures 12, 13, and 14. This stage shows the six, or normal number of, tentacles, although the last two to appear are by no means as fully developed as the others. The

TABLE I

THE DEVELOPMENT OF THE TENTACULAR PATTERN IN THE BUDS OF *Pelmatohydra oligactis*

Description of Stages	Number of Buds Showing Tentacle Pattern	Number Not Conforming to Pattern
First two tentacles opposite each other and in plane at right angles to axis of parent	166	2
Third and fourth tentacles in plane corresponding to axis of parent, the third appearing first and directed toward the apical end of parent	130	10
Fifth and sixth tentacles arising in the angles between the first two and third tentacles	33	1

appearance of the last two tentacles results in a decrease in the angles between the first two and fourth tentacles, with a subsequent equalization of all intertentacular angles about the tentacular ring of the bud (Figs. 13, 14). At this time the stalk of the bud may be partially differentiated (Fig. 12), and the bud itself gives the appearance of a small hydra attached by its basal disk to a larger one.

The observer is particularly attracted by the regularity with which this pattern in tentacular formation appears and with its definiteness as regards the axis of the parent. In the older stages the bud, because of its own muscular activity, may twist about in such a way that the pattern is not in accord with the axis of the parent. Any doubt as to the relationships can be eliminated, however, if the bud is caused to contract, for then it immediately shows the polar relationships we have just described.

During this work hundreds of buds have been observed, and in the vast majority of cases the pattern conformed to that described. An idea of the regularity with which this pattern appears is to be found in Table I, in which the budding hydra were carefully observed under the microscope for the express purpose of recording the pattern. It will be noted that, out of 168 cases, only 2 failed to show the first two tentacles appearing at right angles to the longitudinal axis of the parent. Out of 140 cases, all but 2 showed the third and fourth tentacles arising in a plane passing through the longitudinal axis

of the parent (not shown in table); and in all but 10 cases, the tentacle directed toward the hypostome (third) appeared before the one directed toward the foot (fourth). Of the 34 cases recorded of buds in the fifth- and sixth-tentacle stage, all but 1 case showed the fifth and sixth tentacles appearing in the angles between the first two and third tentacles.

THE GRADIENT IN TENTACLE FORMATION

Although several workers have recorded that pieces taken from different regions of the longitudinal axis of hydra behave differently with respect to tentacle development (Peebles, 1897; Burt, 1925; Weimer, 1928), it appeared desirable, in this work, to attack the problem of rate of tentacle formation by a slightly different procedure. Instead of studying the development of tentacles in pieces of the same length from different levels of the animal, it seemed more logical to make only one cut surface in place of two. This modification was prompted by the work on *Euplanaria* by Child and Watanabe (1935), who noted that the posterior cut surface gave rise to an inhibiting factor which in the more posterior levels of the anterior zooid prevented a normal head from arising on the anterior cut surface of short transverse pieces. (It is not assumed that hydra will behave like *Euplanaria;* but since we do not know what effect, if any, the proximal cut surface in a section of hydra may have, it seemed desirable to eliminate it altogether in these experiments.)

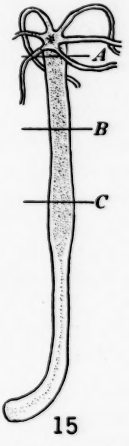


FIG. 15.—Planes of section (*A*, *B*, *C*) made in different lots of *Pelmatohydra oligactis* in determining the gradient in rate of tentacle reconstitution.

Equal numbers of non-budding animals from the same stock were selected for these experiments. The animals were all of approximately the same size, were in good condition, and possessed a well-defined stalk. They were sectioned as shown in Figure 15. Since the highly differentiated stalks are very slow in developing tentacles (some workers have stated that stalks will not develop tentacles), they were not sectioned. The animals in Lot A were sectioned immediately proximal to the tentacles (Fig. 15, plane *A*). In Lot B the cut was made at approximately one-third the length of the body, below the sections made in Lot A (Fig. 15, plane *B*); and in Lot C, approximately two-thirds the length of the body from Lot A sections (Fig. 15, plane *C*).

After the animals were sectioned, the different lots were placed in finger bowls in culture water. They were then kept in the dark at room temperatures for 36 hours before being examined. Examinations were made with the binocular, and the number and size of the tentacles reconstituted in this period were recorded. The tentacles were classified as follows: Class I, bud; Class II, short; Class III, medium; Class IV, long. (This classification was purely arbitrary, since the tentacles classed as "long" were actually less than one-tenth the length of the animal, while the fully developed tentacles may be several times the length of their extended owner.) In order to compare the degree of development of tentacles at the different levels, a "tentacular index" was devised. This consisted of giving the tentacle buds an arbitrary value of 1, the short tentacles a value of 2, the medium tentacles a value of 3, and the long tentacles a value of 4. The number of

tentacles in each stage of development was multiplied by the arbitrary number of that particular stage. The sum of all represented the tentacular index for that particular lot.

In Table II are presented the data for 150 animals, or 50 animals for each type of section. The tentacles are recorded for the lots of 50 rather than for individuals. A comparison of the numbers of tentacles arising from the cut surfaces of the different levels is of interest. In Lot A, 168 tentacles have made their appearance, while Lot B shows 102 and Lot C only 63 tentacles. A comparison of the size of the tentacles shows the same differential, with Lot A far in the lead. The tentacular index gives the combined data of size and number and shows quite clearly the gradient in tentacle development in this species.

TABLE II

NUMBER AND LENGTH OF TENTACLES DEVELOPING AT DIFFERENT LEVELS OF *Pelmatohydra oligactis* AT 36 HOURS AFTER SECTION

(Lots of 50 Animals)

LENGTH OF TENTACLES	NUMBER OF TENTACLES		
	Lot A	Lot B	Lot C
Class I. Buds	24	55	29
Class II. Short	57	35	32
Class III. Medium	68	12	2
Class IV. Longest	19	0	0
Total number of tentacles	168	102	63
Index	418	161	99

In these experiments an effort was made to determine whether or not the tentacles reconstituted in any particular or characteristic pattern. From numerous observations no definite pattern could be found. The tentacles may arise all at the same time or singly. Usually, however, several sizes of tentacles were present about any particular tentacular crown when observed early in reconstitution.

DETERMINATION WITHIN THE BUD

Peebles (1897) noted that pieces of a bud of *Hydra viridis* behaved differently, as regards tentacle formation, in that distal pieces gave rise to tentacles in a shorter period of time and more often than proximal pieces. Goetsch (1920) performed experiments on the same species in which the proximal end of a bud was left attached to the parent. He found a distinct difference between the distal and proximal parts of the bud. These experiments suggest that a certain amount of determination may be present in young buds not yet differentiated into body and stalk. Since the pattern in tentacle development in the bud of *P. oligactis* shows such a high degree of correlation with the longitudinal axis of the parent, and from all appearances seems to be determined in some way by the parent, it seemed of interest to investigate the degree of determination of the bud as a whole at a stage when the tentacle pattern was first becoming evident.

Only animals in good condition with single buds were used in these experiments. The buds had not developed beyond the very early two-tentacle stage and were usually com-

parable to the buds shown in Figures 1, 2, and 3. In no case were they more advanced than the bud shown in Figure 16. These buds were in complete continuity with the parent in that the gastrovascular cavities were continuous and that, when the animals were made to contract, no line of demarcation could be seen between buds and parents.

Distal portions of the bud were removed under a binocular (by a single movement of a small sharp scalpel) when the animals were fully extended. Because of the difficulty in obtaining many animals with single buds at just this stage in development at any one time, the following experiments were performed on lots ranging from 5 to 15 individuals. The sections were made as shown in Figure 16. Two types of operations were performed. In the first type the approximate distal one-third of the bud was removed (Fig. 16, plane *A*), while in the second type the distal two-thirds was removed (Fig. 16, plane *B*). The severed distal tips were not used in these experiments. The parent animals with the attached proximal portions of the buds were placed in culture water in finger bowls and observed after approximately 3 days.

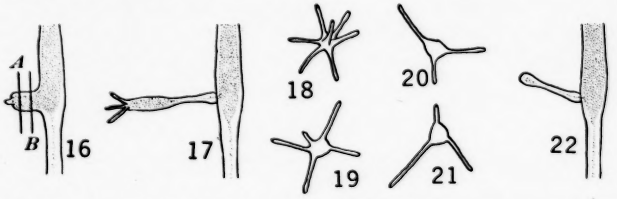


FIG. 16.—Planes of section (*A*, *B*) made in experiments testing the degree of determination in young buds.

FIG. 17.—Development of proximal portion of bud when distal one-third is removed.

FIGS. 18–21.—Different tentacle patterns appearing when distal one-third of bud is removed.

FIG. 22.—Development of proximal one-third of bud. (It commonly gives rise to only a stalk.)

Fifty-two animals were sectioned as shown in Figure 16 (plane *A*), in which only the distal one-third of the bud was removed. In all of these the proximal two-thirds developed into a fairly normal animal possessing hypostome, tentacles, body, and stalk (Fig. 17). In many of these the body appeared somewhat shortened; but since exact measurements were not made, this is somewhat uncertain. The tentacle pattern described above, however, was not found in these mutilated buds. Tentacles of varying numbers, sizes, and shapes appeared about the distal tip of the bud. A few of these different types of tentacle formations are shown in Figures 18–21.

When the distal two-thirds of the buds were removed, a somewhat different picture was presented. The proximal one-third of the bud attached to the parent gave rise to a whole animal only in a limited number of cases. In other cases it gave rise only to a stalk (Fig. 22) or was completely resorbed by the parent. Of the 58 animals sectioned as shown in Figure 16 (plane *B*), only 14 of the buds gave rise to animals with tentacles, while 32 differentiated into stalks alone and 12 were apparently resorbed. These stalks may develop one, two, or even more tentacles after several days; but at the time of observation (approximately 3 days after section) they presented the appearance of differentiated stalks.

The results of these two types of operations are recorded in Table III. From these experiments it would seem that buds in the very earliest stages of tentacle development

are determined as regards other parts (i.e., body and stalk) even though there is no visible differentiation (except first tentacles) and the buds are in complete continuity with the parent.

THE INTERACTIONS OF BUD AND PARENT

Various workers have studied the effects of bud on parent and parent on bud when the region of the parent distal to the budding region has been removed. Goetsch (1917, 1920) has found, in general, that young buds failed to separate from the parent if the distal end of the parent was removed, unless there was sufficient food for them to complete their development. If the parent could furnish sufficient nourishment or the bud was able to take food from the outside, it would sooner or later become detached. Otherwise it remained attached to the parent stalk, eventually forming a stalk of its own.

TABLE III

THE DEGREE OF RECONSTITUTION IN PROXIMAL ONE-THIRD AND TWO-THIRDS OF YOUNG BUDS OF *Pelmatohydra oligactis*

Type of Section	Type of Reconstitution		
	Bud with Tentacles	Stalk Alone	Resorption of Bud
Distal one-third of bud removed	52	0	0
Distal two-thirds of bud removed	14	32	12

Boecker (1921) repeated a number of Goetsch's experiments and found that the bud consistently separated from the parent in the normal manner even though the distal end of the parent had been removed. Weimer (1928) noted that lateral buds may migrate distally and take over, or attain dominance over, the entire piece. In other words, the bud may replace the original apical end. Weimer's results are based on buds which arose after the operations, since he sectioned only the largest animals without buds.

In our experiments testing the effects of bud on parent, and *vice versa*, only animals in good condition and with a single bud were used. The buds were never older than the very early two-tentacle stage (Fig. 2) and were usually without tentacles (Fig. 1). The animals were all approximately the same size and possessed well-defined stalks.

The first set of operations was performed as shown in Figure 23 (plane *A*). The single section was made at a distance of two-thirds the length of the body behind the hypostome. Lots of only 5 or 6 animals were usually cut at the same time, since it was difficult to obtain more at just this stage in budding at any particular time. Observations were made on at least alternate days following section. In all, 43 animals were cut in this manner. Of these 43 cases, 12 developed tentacles at the distal end of the parent piece within 2–4 days after cutting, while the bud completed its growth and differentiation (Fig. 24) and finally detached itself (usually 4–6 days after the operations). Eighteen animals gave rise to a new bud, distal to the old one, by 4 days after section (Fig. 25). In these cases the old bud completed itself and became detached; but tentacles failed to appear at the site of section, at least as long as the second bud remained attached. In 9 of the 18 cases where a second bud appeared, this bud remained attached to the parent.

It either migrated forward and assumed control or it developed its own structures (i.e., foot and stalk) and apparently resorbed the parent (Figs. 26 and 27). In the 9 remaining cases where a second bud appeared, the bud completed its development and became detached, leaving little more than the stalk of the parent behind (Fig. 28). After 8–10 days the parent piece often developed one, two, or even more tentacles.

Of the 25 animals that failed to develop a second bud, 13 failed to develop tentacles at the distal end of the parental part until a much later period. The buds of these 13 all developed completely and became detached, leaving little more of the parent animal behind than the stalk. These parent pieces usually developed a few short tentacles after 8–10 days (Fig. 29).

Thirty-two animals were sectioned as shown in Figure 23 (plane *B*). In this experiment the section was made immediately distal to the bud. Of these 32 cases, 27 of the

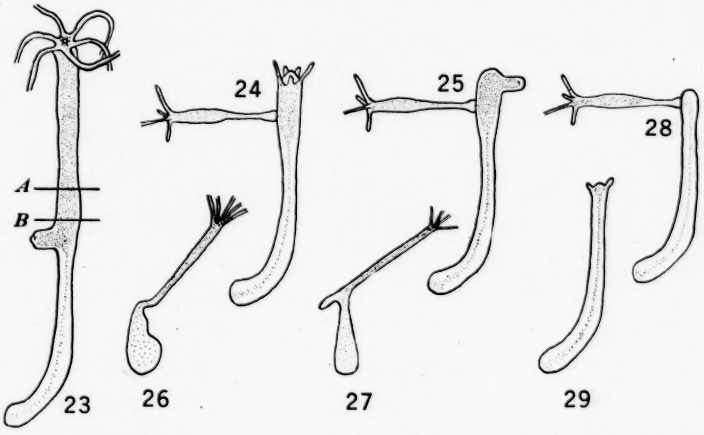


FIG. 23.—Planes of section (*A*, *B*) in experiments testing the mutual effects of actively growing bud and reconstituting parent.

FIGS. 24–29.—Different types of reconstitution and bud development resulting from the foregoing types of section.

buds went to completion and separated, leaving only a stalk and the diminished budding zone behind (Fig. 28). In 4 cases the buds failed to separate but formed a stalk in line with their own axis and eventually resorbed the parental stalk (Fig. 27). In 1 case the bud became the apical end of the parent. In no case, in these experiments, did tentacles appear at the zone of section until long after the bud became detached, although one or more usually did eventually develop. In these experiments one secondary bud arose, which became detached.

It seems to be quite clear from these experiments that an actively growing bud may inhibit the development of tentacles at levels of the parent body a short distance distal to its own level. Also, the bud evidently grows at the expense of the parent and may almost completely, or completely, resorb the parent tissues. Apparently the bud will complete itself and become detached if the parental reserves are sufficient; otherwise it may remain attached to the parent stalk and develop at the latter's expense.

DISCUSSION

It has been found, in general, by the different investigators working with *P. oligactis*, that the first bud usually arises in a certain region of the axis of the parent animal. This region, or budding zone, in large healthy hydra is usually restricted to a certain locus at the proximal end of the body. As additional buds appear, they usually arise at more distal levels than the first. Hyman (1928) points out that the hypostome controls a certain length of the column and that budding does not occur within the limit of control. She shows that, while budding ordinarily occurs at a definite level of the column, the budding zone is not fixed in position, for under proper physiological conditions buds may arise anywhere on the column except within a certain distance of the peristome and foot. Weimer (1928) also interprets budding in terms of the gradient concept and concludes that it results from some degree of physiological or physical isolation from the dominant apical region.

As already noted, the tentacle pattern described above has been observed by others. Koelitz (1910) reviews the literature on development of the tentacles of hydra buds and cites the work of Baker, who, as far back as 1743, noticed that the first two tentacles to appear were directly opposite to one another. Koelitz, himself, worked on the tentacle pattern of four different species. In *H. polypus* and *H. oligactis* he records almost the same pattern as has been here observed, the only differences being the relative times of appearance of the fifth and sixth tentacles. In *H. viridis* and *H. vulgaris* he reports entirely different patterns which apparently have no definite relation to the parent axis.

Koelitz (1910) refers to the side of the bud nearest the hypostome as "dorsal" and to the side nearest the foot as "ventral." In this terminology we may describe the first two tentacles in the *Pelmatohydra* bud which arise at the same time, as arising laterally. The third tentacle arises on the dorsal side and is usually ahead of the fourth tentacle, which arises directly opposite, on the ventral side. The fifth and sixth tentacles appear on the dorsal side of the bud in the angles between the first two and third tentacles.

The experiments on the gradient in tentacle formation show that the number of tentacles appearing and the growth in length in a given time decrease from distal to proximal levels. With these data at hand it is readily seen why the dorsal side of the bud should be more active than the ventral. *The dorsal side is derived from a higher level of the parent gradient.* We have here then an example of a developmental pattern derived, at least in part, directly by the bud from its parent.

Although the pattern of tentacle development may be traced, in part, directly to the parent, conditions in the bud are evidently also concerned in localization of the tentacles. Although the dorsal side of the bud supposedly represents a higher gradient level than any other part of its circumference, it does not give rise to a tentacle first. The tentacle pattern indicates a delicate balance between conditions in the parent body and the bud. Any part of the bud circumference is capable of giving rise to a tentacle, but the first tentacles appear simultaneously at loci which are as far from each other as possible and alike with respect to the parental gradient. The following interpretation suggests itself. The bud apparently originates in consequence of some degree of physiological isolation from parental dominance, but in the early stages of the bud this physiological isolation is not yet complete. The effect of parental dominance is to prevent or retard development of a new apical region. If this dominance is slightly more effective dorsally than ventrally, in the bud it may be sufficient to retard tentacle formation dorsally but not

sufficient to prevent such development laterally. As the bud elongates further, its distal end becomes more completely isolated from parental dominance, and traces of the dorsoventral gradient which remain in the bud determine the third tentacle dorsally earlier than the fourth on the ventral side and also determine the fifth and sixth tentacles dorsally. The fact that the fifth and sixth tentacles arise dorsally indicates there is still a dorsoventral physiological differential in the bud. On the other hand, the fact that either the right or left of these tentacles may appear in advance of the other or that the two may appear simultaneously suggests that slight local differences about the circumference of the bud are also concerned in determining the time of appearance. It appears possible to account for the tentacle pattern only in some such terms as these. In other species of hydra parental dominance and the parental gradient may be less or not at all concerned in the tentacle pattern of the bud, and the order of tentacle appearance may be determined by incidental factors acting directly on or in the bud and may differ in different individuals.

Various workers have presented evidence of axial gradients of various kinds in freshwater hydra. Distal-proximal gradations in rate and number of tentacles reconstituting on pieces from different levels of the body axis have been noted (Peebles, 1897; Burt, 1925; Weimer, 1928). Distal-proximal gradations in disintegration rate in toxic solutions and in rate of potassium permanganate reduction have been found (Child and Hyman, 1919). Hinrichs (1924) demonstrated a distal-proximal gradient of disintegration in hydra by means of photolysis. Recently a like gradation in methylene blue reduction has been recorded (Child, 1934). All the evidence points quite definitely to a differential in rate of activity along the polar axis. In hydra the locus of highest activity of the primary gradient has invariably been found to be the apical region, which is represented by the tentacular ring and hypostome.

But secondary regions of high activity have also been found to be present in the living animal. The susceptibility method has revealed that the foot or the adhesive basal disk, loci of high digestive activity, and regions of strong muscular contraction show greater susceptibility (Child and Hyman, 1919). Weimer (1928) has found that sections from the budding region may reconstitute a greater number of tentacles than like sections from regions immediately distal.

In our experiments on rate and number of tentacles developing from the different levels, only the primary gradient appeared. We failed to obtain the rise of activity at the budding region which Weimer records. It must be pointed out, however, that in our experiments only one cut surface was made. Also, the animals used in these experiments were not budding animals, nor did buds arise in a single case during the course of the experiment.

It has been mentioned along with the experiments that distal and basal pieces of young buds of *H. viridis* have been found to behave differently in that distal ends were more active in tentacle formation (Peebles, 1897; Goetsch, 1920). Distal regions of the buds of *P. oligactis* have likewise been found to be more susceptible than basal regions(Child and Hyman, 1919). Our experiments in which the distal one-third and distal two-thirds of the very early bud (not older than the early two-tentacle stage) were removed show some degree of determination in the bud before this early stage and show that it is already an individual, although still in complete continuity with the parent. When the distal one-third was removed, the remaining two-thirds completed its development in all cases. But after removal of the distal two-thirds, the basal third remaining attached

to the parent, though not visibly different from the middle third, gave rise, in 32 of the 58 cases, to a stalk only; and tentacle development, when it occurred (14 cases), was slow.

The tentacle pattern in these cases of reconstitution from basal two-thirds or one-third of the bud did not show any definite relation to the parental axis and differed in different individuals. This is not surprising, for the injury resulting from section may determine local differences in activity in different parts of the circumference of the mutilated bud which overbalance any effect of parental dominance and gradient. Moreover, the reconstitutional activity following section and the elongation of the mutilated bud may make physiological isolation complete before the new tentacles appear, so that in these cases the tentacle pattern is determined entirely by local incidental factors.

Goetsch's experiments (1917, 1920) in which the distal end of a budding hydra was removed indicated that the bud was in some way prevented, by this operation, from separating from the parent. Boecker (1921) found that the removal of the distal end of the budding hydra in no way prevented the separation of the bud from the parent. Weimer (1928) reported that budding was most often followed by bud separation, in experiments on hydra at the point of budding, if the distal one-half to two-thirds of the body and the stalk were removed. If the stalk remained, the percentage of bud separation was reduced to zero. He also stated that if the stalk remained attached to the piece the bud migrated distally and took control of the proximal body-level and the stalk already formed. Weimer tabulates only 6 animals in which as much as the distal one-half was removed and the stalk left attached. Of these 6 animals, none formed buds which became detached.

Our experiments in which distal ends of animals with young buds were removed show quite definitely that the bud may complete its development and become detached. Not only may the primary bud complete its development but a secondary bud which arose after section may also complete its development and become detached. Observations on hydra with actively growing buds in which the distal end of the parent has been removed show clearly a dwindling in size of the parent tissue as the bud grows. Since the bud is a determined, although not visibly differentiated, individual, it appears not unlikely that it may grow by a process involving the breakdown of parent cells and a subsequent reassimilation of the nutritive elements into the tissues of the bud. Moreover, the bud seems usually to live and grow more at the expense of the proximal part of the body proper than of the stalk, though under certain conditions it may resorb the stalk. If the tissue of the parent is insufficient to allow the bud to complete its development, it may remain attached for a considerable time to little more than the parent stalk, for apparently the stalk does not yield so freely to the demands of the bud.

An actively growing bud near the region of section apparently prevents, through its activity, the reconstitution of parental tentacles. If, however, the section is far enough removed from the bud, the tentacles will be reconstituted on the parent as usual. In a few cases the bud has been noted to take over and control the parent piece by shifting in such a way as to become its apical end. Usually, however, after a bud is once started, it will complete itself and become detached if the parental reserves are sufficient.

SUMMARY

1. The developing bud of *P. oligactis* presents a regular pattern in tentacular appearance which shows a high degree of correlation with the axial gradient of the parent.

a) The first two tentacles arise opposite each other at the same time and grow at right angles to the polar axis of the parent.

b) The third and fourth tentacles lie in the same plane as the polar axis of the parent. The third tentacle is directed toward the hypostome of the parent and usually is well in advance of the fourth tentacle, which is directed toward the basal disk.

c) The fifth and sixth tentacles arise at the same time or singly. They arise in the angles between the first two and third tentacles.

2. Single sections made at three different levels of three different lots of animals show a basipetal decrease in rate of growth and in number of tentacles developing in a given period of time (36 hours).

3. Experiments involving the removal of the distal one-third and the distal two-thirds of young buds indicate that a high degree of determination of body and stalk is already present at the early two-tentacle stage.

4. Experiments on hydra with young buds in which the distal two-thirds or more of the parent was removed indicated that the bud may inhibit reconstitution in the parent for as long as it remains attached if it is near the plane of section.

5. In experiments involving the removal of the distal end of the parent, it has been found that a bud, once started, will usually complete its development and become detached if the parental reserves are sufficient. Lack of parental reserves may cause the bud to remain attached and ultimately to resorb the parent stalk. In other cases the bud may migrate distally and assume direct control.

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CERTAIN PHYSIOLOGICAL EFFECTS OF X-RAYS ON EUPLANARIA DOROTOCEPHALA. II. REGENERATION

(Seven figures)

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SOME differences of opinion exist relative to the ability of animals to regenerate tissues following X-ray irradiation. The experiments discussed in the present paper were performed as an attempt to answer this question, in part at least, with respect to the flatworm, *Euplanaria dorotocephala*. The procedure followed was to expose a number of these animals to a variety of X-ray dosages, and then to cut them, at different intervals following the irradiation, into pieces which, if cut from unirradiated animals, would regenerate normally all, or at least nearly all, their lost parts.

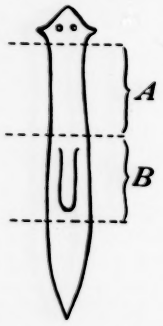

FIG. 1.—Diagram of *Euplanaria dorotocephala* showing regions cut to give A and B pieces.

The animals used were taken from a spring near Rockford, Illinois. Before experimented upon, they were kept in the laboratory several months in order that they might become acclimated to the laboratory conditions, and also in order they might become more physiologically uniform. Two weeks prior to the irradiation a stock pan was set aside, and the animals in it were no longer fed. On the day of the irradiation, 480 animals, measuring between 18 and 20 mm. in length when crawling on a surface film of water, were selected from the starvation stock. These 480 animals were then subdivided into six lots of 80 animals each. Of the six lots obtained in this manner, one of them, Lot I, was kept for control purposes; whereas the other five, Lots II, IV, VI, VIII, and X, as they were designated, were irradiated 2, 4, 6, 8, and 10 minutes, respectively. The X-ray machine used was operated at all times at 85 peak kv. (alternating potential) and at 5 m.a. The animals irradiated were in each case exposed at a distance of 35 cm. from the target. Thus the only variable was the length of the exposure. As measured at various times during the course of the experiment, the tube, under the conditions given, delivered approximately 86.4 roentgen units per minute.

After the irradiations each of the six lots was subdivided into four smaller lots of 20 animals each. The animals of one lot of 20 animals of each exposure and of one control lot were then cut into A and B pieces (see Fig. 1). The remaining 360 animals were cut into similar pieces on the second, fourth, and sixth day following the irradiation; i.e., one lot of each exposure was cut on the second day, one of each on the fourth day, and one of each on the sixth day.

Following the cutting of the animals, each lot of 20 pieces was examined daily under a dissecting microscope over a period of 6 weeks; and daily records were kept of the extent

of the regeneration shown by each piece, as well as of the visible disintegration effects which appeared as the result of the irradiation.

Figure 2 gives the maximum degree of regeneration reached by each lot. The point plotted for each lot represents the highest mean of the following values assigned daily to the different pieces within the lot: a value of 100 to a piece which had regenerated normally all head tissues; a value of 80 to a piece which had regenerated all head tissues except those of the eyes; a value of 60 to a piece which showed abnormal auricles as well as abnormal eyes; a value of 40 to a piece which had regenerated some head tissues but lacked eyes; and a value of 20 to a piece which had not regenerated any tissues.

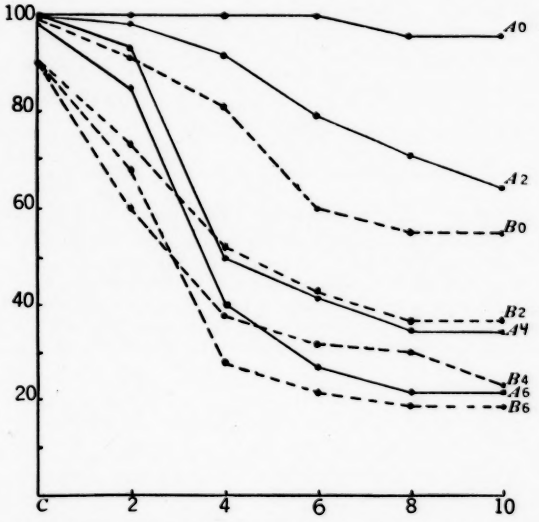

FIG. 2.—The maximum degree of regeneration shown by lots of 20 A and B pieces of *Euplanaria dorotocephala* following five different X-ray dosages. The ordinates represent the degree of regeneration. A value of 100 represents normal regeneration of all parts; a value of 20, no regeneration. The abscissas are minutes exposed. An exposure of 1 minute is equivalent to about 86.4 roentgen units. *C* stands for control. The letters and numbers on the curves represent, respectively, the level of piece and the length of time in days after the irradiation when the pieces were cut; 0 means immediately after the irradiation.

Although Figure 2 gives the maximum degree of regeneration shown by each lot, it does not show when this maximum was reached; neither does it show the degree of the disintegration which appeared in some of the irradiated pieces. An attempt is made to give these results in Figures 3, 4, 5, 6, and 7. The points plotted in these graphs are the means of arbitrary values representing the combined effect of the regeneration and disintegration processes at the end of each 7-day period following the cutting of the pieces. The values assigned for the determination of these means are as follows: a value of 100 to a piece which had regenerated normally all its head tissues and showed no disintegration; a value of 70 to a piece which had regenerated tissues which included one or two eyes but which did not show the normal relationships of all the parts, or to a piece

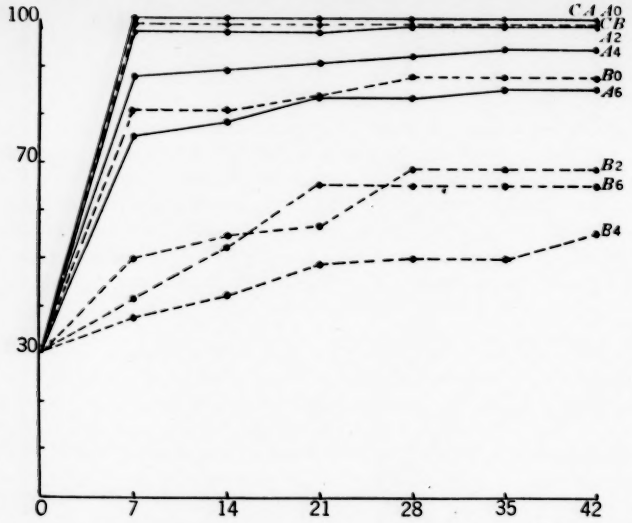

FIG. 3.—Graph indicating the degree of regeneration and disintegration shown by lots of 20 A and B pieces of *Euplanaria dorotocephala* following an X-ray exposure of 2 minutes. An exposure of 1 minute is equivalent to about 86.4 roentgen units. Ordinates, arbitrary values. A value of 100 represents a normal head regenerated and no disintegration. A value of 0 represents complete disintegration. Abscissas, days after the cutting of the pieces. The letters *A* and *B* on the curves represent body-levels of the pieces, and the numbers 0, 2, 4, and 6 represent the length of time in days between the irradiation and cutting of the pieces; 0 means immediately after the irradiation. The curves *CA* and *CB* represent the control lots.

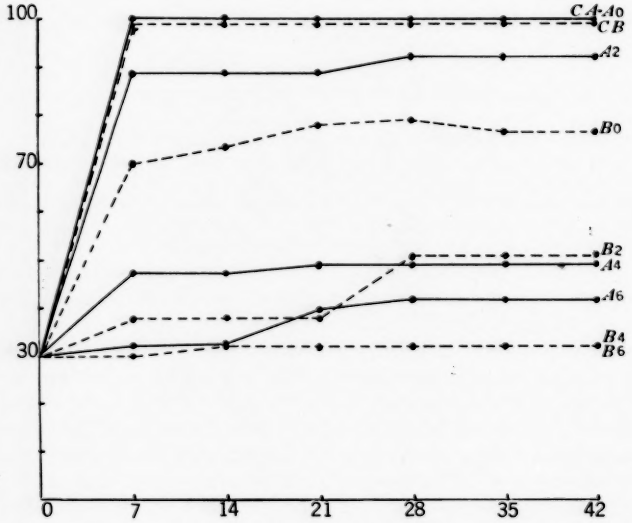

FIG. 4.—Graph indicating the degree of regeneration and disintegration shown by lots of 20 A and B pieces of *Euplanaria dorotocephala* following an X-ray exposure of 4 minutes. (Ordinates and abscissas are the same as in Fig. 3.)

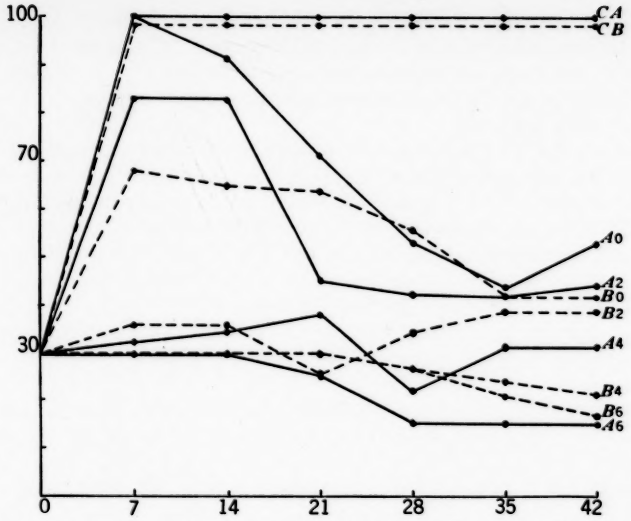

FIG. 5.—Graph indicating the degree of regeneration and disintegration shown by lots of 20 A and B pieces of *Euplanaria dorotocephala* following an X-ray exposure of 6 minutes. (Ordinates and abscissas are the same as in Fig. 3.)

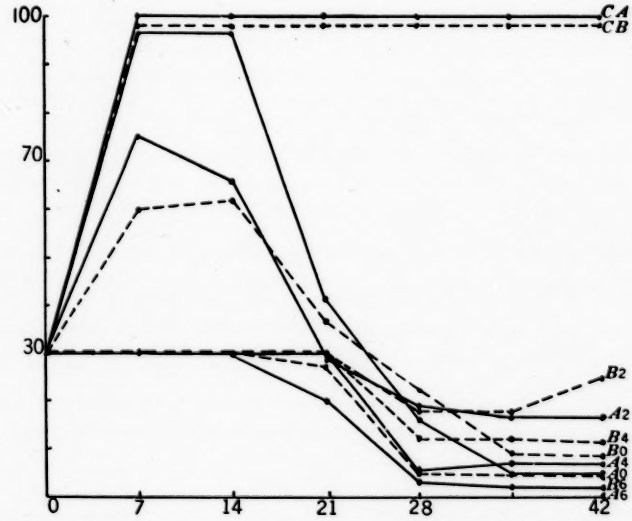

FIG. 6.—Graph indicating the degree of regeneration and disintegration shown by lots of 20 A and B pieces of *Euplanaria dorotocephala* following an X-ray exposure of 8 minutes. (Ordinates and abscissas are the same as in Fig. 3.)

which had regenerated a normal head but which showed disintegration effects in the head region; a value of 30 to a piece which had regenerated some head tissue but lacked eyes, or to a piece which had regenerated tissues including an eye or eyes but had lost the new tissues, at least as far as and including the eyes; and a value of 0 to a piece which had disintegrated completely.

In Figures 3–7 the steeply rising parts of the curves represent the early stages of head regeneration. The fall to the right of the maximum which appears in many of the curves indicates that regeneration is followed by more or less disintegration. The curves which show no steep rise and fall represent either inhibited regeneration or regeneration plus more or less disintegration.

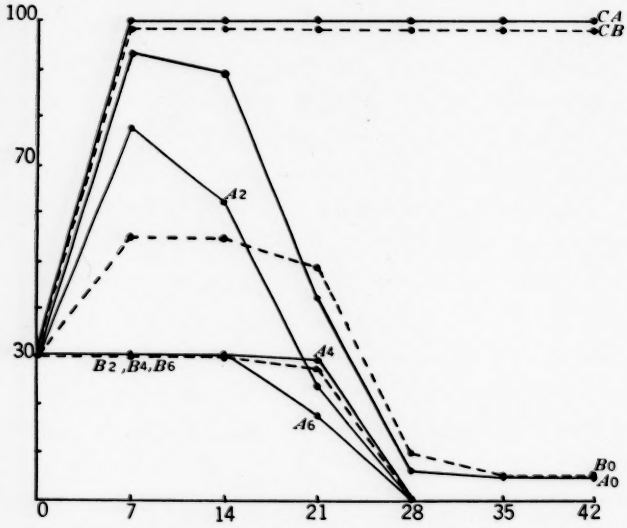

FIG. 7.—Graph indicating the degree of regeneration and disintegration shown by lots of 20 A and B pieces of *Euplanaria dorotocephala* following an X-ray exposure of 10 minutes. (Ordinates and abscissas are the same as in Fig. 3.)

Comparison of Figures 3–7 (Fig. 3, 2 minutes; Fig. 4, 4 minutes; Fig. 5, 6 minutes; Fig. 6, 8 minutes; Fig. 7, 10 minutes) indicates that with the shorter periods of irradiation there is little or no disintegration (Figs. 3 and 4). With increase in period of irradiation, regeneration during the earlier part of the latent period decreases somewhat and disintegration following the latent period shows a marked increase.

Comparison of the curves in each figure shows that more regeneration occurs in A than in B pieces but that, with the longer periods of irradiation and longer time between irradiation and cutting, A pieces finally disintegrate to a greater extent than B pieces (Fig. 5, *A6*, *B6;* Figs. 6 and 7).

DISCUSSION

It is evident from the data presented in Figure 2 that cut pieces of *E. dorotocephala* will regenerate tissue following X-ray irradiation. However, it is also evident that the

degree of regeneration decreases with each increase in the length of time which passes between the irradiation and the cutting of the pieces. Furthermore, the data also show that anterior A pieces will regenerate initially more tissue than posterior pieces given the same dosage, although the former, after a latent period of 10 or 12 days, will show a greater degree of disintegration.

That cut pieces of *Euplanaria* will regenerate tissue following X-ray irradiation was not unexpected. In previous experiments (Strandskov, 1934) it was observed that fission pieces from irradiated whole worms of this species regenerated some head tissues. The degree of regeneration which followed the 10-minute dosages, especially of the A pieces cut immediately following the exposure, was, however, surprisingly great. It will be noted that it almost equaled that of the control (see *Ao*, Fig. 2).

Although A pieces which were irradiated 10 minutes and were cut immediately after the exposure regenerated almost as much tissue as did similar pieces irradiated 2 minutes, all the other pieces showed a decrease with each increase in dosage. The exact relationship which obtains between the degree of regeneration and the dosage cannot be determined definitely from the data, but it is probably not exactly a linear one; an inverted S curve is suggested by the curves *Bo*, *A4*, *A6*, and *B6* of Figure 2. Packard (1931), irradiating the eggs of *Drosophila*, obtained definitely such a curve when he plotted the percentage of eggs killed against dosages of X-rays measured in terms of roentgen units.

The greater degree of regeneration shown by pieces cut immediately after the irradiation than by pieces given the same dosage but cut at a later time is of interest. It is undoubtedly an illustration of the well-known delayed effects of X-rays. In previous experiments (Strandskov, 1934) it was found that visible disintegration effects due to X-rays do not appear on whole *Euplanaria* before the tenth or twelfth day following the irradiation. Thus, with pieces cut immediately after the exposure, a considerable degree of regeneration takes place before the action of the rays becomes effective. An inhibiting effect, however, appears before the tenth or twelfth day, because, as shown by the curve *B6* of Figure 2, posterior pieces irradiated 10 minutes and cut on the sixth day show no regeneration whatsoever. Whether a strong-enough dosage can be given which will inhibit all regeneration of pieces cut immediately after the exposure, the present data do not show; but the possibility is suggested by the trend of the curve *Bo* of Figure 2.

The greater degree of regeneration shown by irradiated anterior A pieces than by the posterior B pieces given the same dosage may seem contradictory to the general conclusions of Child, namely, that physiologically active tissues are affected by external agents, in sufficiently high concentration or dosage, to a greater extent than are those less active. However, when one considers the delayed effects of X-rays, the results are probably not in disagreement with the general conclusion just mentioned. Child and others have shown that untreated anterior pieces of *Euplanaria* have a higher head frequency than posterior pieces (see especially the recent paper of Child and Watanabe, 1935). This fact is even shown by the control lots of the present experiments (see ordinates of *C*, Fig. 2.) Furthermore, Watanabe (1935) has shown that the rate of development of head tissue in untreated *Euplanaria* decreases from anterior to posterior levels. Thus, with a latent period of at least a few days one should expect a greater initial degree of regeneration shown by pieces from the anterior levels. That the anterior pieces are actually affected to a greater extent by the X-rays is shown by the results

obtained after the latent period. The curve *Ao* of Figure 6 rises high above *Bo* during the latent period but dips below it at the end of the fourth week, and stays lower after that time.

As was observed in previous experiments (Strandskov, 1934), and as shown again in these experiments (see *Ao*, *A2*, *A4*, and *B2* of Fig. 5), pieces which have lost some of their tissues as the result of disintegration due to the effects of X-rays may regenerate new tissue after the disintegration effects have worn off. Furthermore, as was also observed in previous experiments and verified again in these, pieces which survive the longer irradiations, after a partial disintegration, will regenerate head tissue when their anterior marginal tissues are removed. Thus, it does not seem probable, as has been suggested by some, that pieces of *Euplanaria* or *Planaria*, which have completely lost the ability to regenerate new tissue due to the effects of X-ray irradiation, can be obtained and kept alive for any considerable length of time.

SUMMARY

1. Four hundred flatworms of the species *E. dorotocephala* were exposed to various dosages of X-rays and then cut into A and B pieces in order to determine the degree of regeneration which would follow such irradiation.

2. Most of the irradiated pieces regenerated some tissues.

3. There was a decrease in the maximum degree of regeneration with each increase of dosage; also with each increase in interval between the irradiation and the time of the cutting of the pieces.

4. Anterior A pieces regenerated more tissue immediately after the irradiation than did the corresponding more posterior B pieces. This is undoubtedly a consequence of the delayed effects of X-rays.

5. Pieces given the longer exposures showed visible disintegration effects and some times disintegrated completely. However, such pieces, if cut soon after the irradiation, regenerated in most cases some new tissues before they started to disintegrate.

6. Anterior A pieces, after the latent period, disintegrated to a greater extent than did corresponding more posterior B pieces.

7. Pieces which had regenerated some tissues and had lost them as a result of disintegration due to X-rays, but which did not disintegrate completely, began again, in some cases, to regenerate lost parts.

8. Although it was found that X-ray dosages can be given which will prevent pieces of *Euplanaria* from regenerating any tissues immediately following X-ray irradiation, no piece which had permanently lost the ability to regenerate was obtained and kept alive for any considerable length of time.

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EFFECTS OF TEMPERATURE ON MOVEMENTS OF EMBRYOS (ACRIDIDAE, ORTHOPTERA)[1, 2]

(Eight figures)

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THE rhythmic contractions of the lateral body walls in grasshopper embryos (*Melanoplus differentialis*) were observed by Nelsen (1931), who suggested that they represented rudiments of heart beats. These contractions have been employed as physiological indicators by Slifer (1934), working on the influence of anisotonic solutions, and by Walker (1935), in his study on carbon dioxide effects. It was, therefore, considered desirable to study the rate of behavior of these movements at different temperatures from the time of their initiation, through the period of heart formation, and up to the time of hatching.

DESCRIPTION OF MATERIALS AND METHODS

Eggs of two species of grasshoppers, *Melanoplus differentialis* and *M. femur-rubrum*, were used in the present study. Information regarding the collecting, handling, and care of these insects and their eggs may be obtained from the accounts of Carothers (1923), Bodine (1925), and Slifer (1932*a*). The egg pods were kept at 25° C. for from 21 to 25 days after laying. Eggs of *M. femur-rubrum* were then ready for use (or may be placed in the icebox until desired). *Melanoplus differentialis* eggs, on the other hand, characteristically enter a period of non-development known as diapause (Bodine, 1932), and must be left at 0° C. or thereabouts for about 6 weeks before development will proceed farther upon return to a higher temperature. Long exposure to low temperatures at this stage does not seem to injure the embryo. The eggs of both species have been kept at 0° C. for 3 years; and their development, on removal to 25° C., is normal.

The grasshopper embryo and yolk are surrounded by two membranes. The outer covering, an opaque layer known as the chorion, may be easily peeled away, whereupon the embryo becomes clearly visible through the transparent second layer or cuticle (Slifer, 1932*b*). When the eggs from which the chorion had been removed were exposed to 0° C., development on returning to room temperature was somewhat retarded. However, an exposure of the eggs to 5° C. seemed to exert no harmful effects. Accordingly, such eggs were kept at 5° C. until desired for use.

Eggs at the correct morphological stage (Stage 1, see below) were arranged in rows on black absorbent cotton (about 0.5 cm. thick) on a container measuring 6×6×4 cm. This was covered with a glass plate, and placed in a water bath at the desired temperature. At temperatures above 26° C. the inner surface of the glass was coated with a thin film of glycerine and water. This prevented formation of water vapor on the glass through which observations were to be made.

[1] Aided by grant from the Rockefeller Foundation for research in cellular physiology.

[2] The author wishes to express his appreciation to Dr. J. H. Bodine for suggesting the problems, and for his interest and help at all times.

A binocular microscope (20 diameters) was used for all observations. A stop watch graduated in 0.1 seconds was used in order to determine the rate of lateral body-wall contractions. The time required for from four to forty contractions (the number depending upon the rate of movement) was noted and the result then converted to contractions per minute. Each reading was carefully checked. For illuminating the eggs, a 9.9-volt flashlight bulb was used. This was placed in a two-cell flashlight and operated from the house current by means of a transformer. The size and ease of handling of this illumination source made it possible to direct the beam of light on any egg desired. Further, the heat radiated from this size electric bulb is so small as to be completely negligible.

The first movements of the embryo are evident just before blastokinesis. These are at first quite feeble, but slowly increase in intensity, soon forcing the embryo out of its position in the yolk and causing revolution around the posterior end of the egg (Slifer, 1932*b*). As a result the embryo is oriented with its head pointed away from the micropyle end of the egg. This stage will be designated as "Stage 1," or the post-blastokinesis stage, in this paper. The eyes are plainly visible and are to be found on either side of the egg, about half-way between the two ends. Contractions of the lateral body walls are clearly observable and may be described as a peristaltic wave traveling from the posterior to the anterior ends of the lateral walls.

The embryo grows rapidly in length; and this, together with the upward growth of the lateral body walls, results in the complete surrounding of the yolk. This process, the completion of which will be designated as "Stage 2," is known as yolk engulfment. At this stage the lateral walls have fused over the top of the yolk, but the rhythmical contractions continue just ventral to them where the heart is being formed. Details of these changes may be found in the papers of Nelsen (1931) and Slifer (1932*a*, 1932*b*).

The fully formed heart of the grasshopper is a simple dorsal tube. The earliest rudiments of the insect heart, acocrding to Korotneff (1883), appears as a longitudinal strand of cells (cardioblasts) located along the upper and external border of the dorsal subdivisions of the primitive segments. These heart rudiments are carried dorsad by the lateral walls (Nelsen, 1931), reaching their ultimate position when the walls have fused.

Shortly after yolk engulfment, pigment dots appear on the pronotum. These dots are elaborated in definite order from embryo to embryo, finally forming the color pattern on the pronotum of the hatching insect. This gives one an additional means of determining the stage of development. The appearance of a row of pigment dots (eight in number) along the anterior border of the primitive pronotum will be known as "Stage P." The whole embryo occasionally undergoes struggling peristaltic movements. These become more and more violent until finally the cuticle, being considerably weakened (probably by action of a hatching enzyme) (Williams, 1933), splits and the embryo works its way out of the shell.

RESULTS

EGGS KEPT AT CONSTANT TEMPERATURES FROM STAGE 1 TO HATCHING

Figures 1 and 2 show graphically the lateral-wall/heart-rate rhythm in contractions per minute plotted against time for embryos incubated at the temperatures indicated. At each temperature, two lots of at least 120 eggs were used. Readings of contraction rates and developmental stages were taken twice daily for the 30° C. and 35° C. groups, daily for the 25° C. lots, and on alternate days for the eggs kept at 20° C. The number

of days from Stage 1 to hatching at each temperature, together with the corresponding rate and the Q_{10} values, are given in Table I.

No record of the movements during blastokinesis has been included in any of the graphs, since at this time, especially at lower temperatures, such movements are quite irregular and frequently stop. They have never been observed at temperatures below 18° C. When present, however, the movements tend to be slightly more rapid than those after blastokinesis.

TABLE I

TOTAL NUMBER OF CONTRACTIONS AND DEVELOPMENTAL TIME AT VARIOUS CONSTANT TEMPERATURES

Days from Stage 1 to hatching for eggs of *Melanoplus femur-rubrum* and *M. differentialis* kept at constant temperatures. Rate in reciprocal days. Q_{10} for each temperature range. Total contractions performed by embryo from Stage 1 to hatching determined from area under each curve in Figures 1 and 2.

Temperatures	Days	Rate	Q_{10}	Total Contractions
	Melanoplus femur-rubrum			
20° C.	24	0.0416	4.0	1.31×10^6
25	12	0.0832	1.76	1.17×10^6
30	9	0.1110	1.26	1.19×10^6
35	8	0.125		1.33×10^6
	M. differentialis			
20	27	0.0370	3.22	1.59×10^6
25	15	0.0663	1.57	1.64×10^6
30	12	0.0832		1.57×10^6

The first rhythmic phase (see Figs. 1 and 2) may be considered as that from Stage 1 to Stage 2. During this time the rate of contraction slowly increases. Certain anomalies may be found, which will be discussed later. Following this increase, a second phase appears in which the rate remains more or less stationary. It will be remembered that the lateral walls have fused and that the heart is being formed at this time. After its completion, there is a rapid increase in contraction rate, continuing until nearly the time of hatching. Before hatching, the rate becomes fairly constant. The heart rate of the embryo while struggling in the egg does not increase above that in the same embryo while resting.

At 35° C. abnormal conditions appeared in the development of *Melanoplus differentialis*. It was impossible to keep the chorionless eggs from becoming soft even though they were partially immersed in water. After a few days at this temperature the embryos

"popped" out of the shells with no pigment development on the pronotum. Such animals never molted as do normal individuals immediately after leaving the eggs. In the case

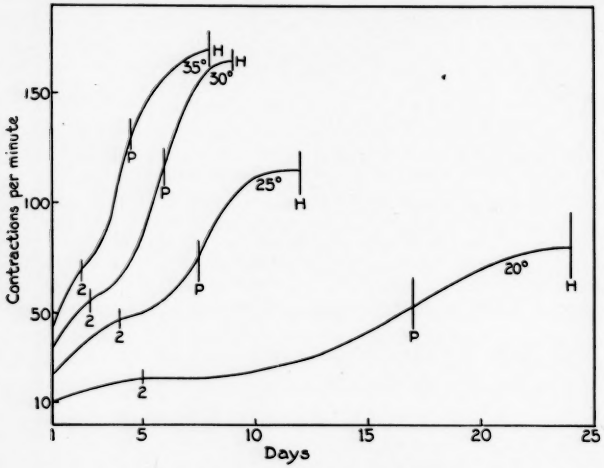

FIG. 1.—Contractions per minute—days development for *Melanoplus differentialis* at constant temperatures. Solid line represents path followed by over half of 240 embryos; spread of remainder indicated by vertical lines at Stage 2 (yolk engulfment), P (pigmentation on pronotum), and H (hatching).

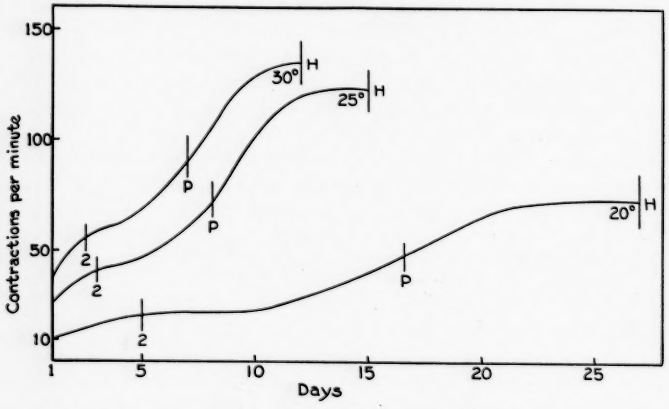

FIG. 2.—As in Figure 1, but for *Melanoplus femur-rubrum*

of *M. femur-rubrum*, development was completed at 35° C.; and the embryo molted in most cases. Here again, however, the embryos at the time of hatching were less pigmented than those kept at lower temperatures.

The removal of the chorion does not affect the time needed for hatching. At all temperatures a group of eggs with intact chorions were run. A number of these eggs (usually 10) were peeled every day on which observations were made, and no difference in contraction rates or developmental stages could be found when these were compared with the chorionless eggs.

2. THE EFFECT OF TEMPERATURE ON CONTRACTION RATES

The effect of temperature on the rate of lateral wall and heart contractions was determined for a temperature range from 12° C. to 40° C. Embryos of both species were carried over this temperature range at Stages 1 and 2 and at hatching. It was found that the hearts of embryos kept at lower temperatures from Stage 1 to the time of hatching

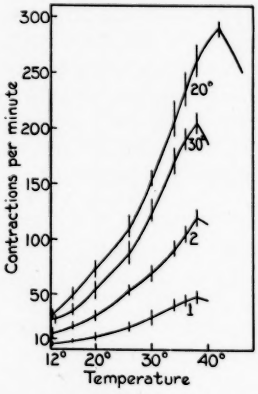

FIG. 3

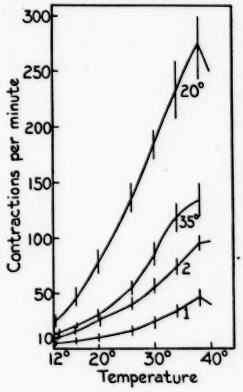

FIG. 4

FIG. 3.—Contractions per minute—temperature for *Melanoplus differentialis*. 1, embryos at Stage 1 (post-blastokinesis). 2, Stage 2 (yolk engulfment). 20°, at hatching, incubated from Stage 1 at 20° C. 30°, at hatching, incubated from Stage 1 at 30 °C.

FIG. 4.—As in Figure 3, but for *Melanoplus femur-rubrum*.

beat more rapidly than those incubated at higher temperatures. Accordingly, separate observations were made on hatching *Melanoplus differentialis* incubated at 20° C. and 30° C., and on hatching *M. femur-rubrum* which had been kept at 20° C. and at 35° C.

The results are presented graphically in Figures 3 and 4. The maximal rate of contraction for all lots was at 38° C., except for the *M. differentialis* group incubated at 20° C. There the thermal maximum was increased to 42° C.

3. CONDUCTIVITY RATE OF THE CONTRACTION IMPULSE

A lateral wall (or heart) contraction is initiated at the posterior end of the embryo and moves anteriorly. The rate at which the contraction travels was determined by observing the time taken for a contraction wave to traverse a certain distance as measured by an ocular micrometer. The results are shown in Figure 5. At the same temperature, a more rapid rate of conduction is seen for Stage 2 (about 7.3 mm. per second), when the heart is being formed, than for the simple lateral body-wall move-

ments (about 2.8 mm. per second). This is still lower than 20–40 mm. per second, which is claimed by Clark (1927) to be the rate of conduction in the hearts of many insect larvae and adults.

4. DIASTOLE/SYSTOLE (D/S) RATIO

The ratio of the time during which there is no lateral wall movement to the time spent in motion, viz.,

$$D/S = \frac{\text{Time relaxed}}{\text{Time movement}},$$

is known as the diastole/systole ratio. Observations were made by timing the two phases of a contraction at a definite point (usually the mid-point) on the lateral walls. Figure 6 shows the D/S ratio for the temperatures 12°–38° C. for *Melanoplus differentialis* at Stage 1. At the lower temperatures, less than one-fourth of the time is spent in movement. As the temperature increases, more time is used for contraction. The lowest ratio, about 0.5, was obtained at 34° C., where a section of lateral wall is relaxed only about one-third of the time during a contractile cycle. Above 34° C. the resting phase becomes relatively longer (i.e., D/S is greater than 1), although the actual rate of contraction continues to increase.

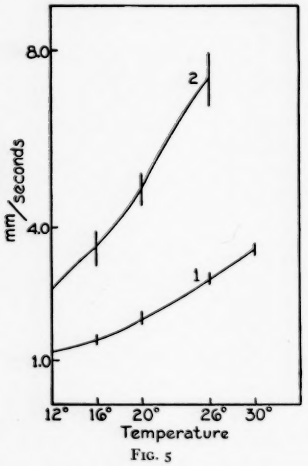

FIG. 5

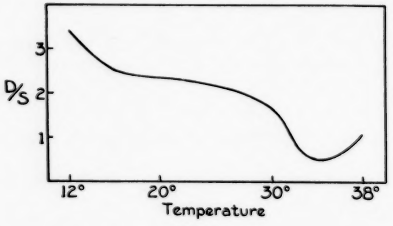

FIG. 6

FIG. 5.—Rate of conduction of contraction impulse at different temperatures. *Melanoplus differentialis* embryos at Stage 1 (curve 1) and Stage 2 (curve 2).

FIG. 6.—Diastole/systole ratio at different temperatures. *Melanoplus differentialis* at Stage 1.

DISCUSSION

The rate of initial contractions of the lateral walls of the grasshopper embryo is quite low. Muscle cells are not yet completely differentiated (Slifer, personal communication). In general, the first contractions of future heart regions are of a simple myogenic type. Crozier and Stier (1926) claim that the initial contractions are myogenic in *Limulus* embryos. Moreover, Patten and Kramer (1933) state that contraction commences in the chick heart "before any neuroblasts approach the heart, and before there is any recognizable histological differentiation of cardiac muscle."

Nelson (1931) suggested that the lateral body-wall contractions are resolved into those of the grasshopper heart when it is formed. It is possible to follow the contractions without a break to the time of hatching. In fact, as the heart is formed, and before its

rudiments have been carried to their final position, it is passively moving with the lateral walls.

During the period of yolk engulfment (from Stage 1 to Stage 2) a number of interesting anomalies occasionally appear in the type of contractions. One of these is the reversal of direction of beat, in which the first indication of contraction occurs at the anterior end and migrates posteriorly. The contractions may become normal without missing a beat. Other cases have been noted in which change to the normal type was preceded by a period in which the movements alternated as to their origin, or in which both ends contracted simultaneously. This reversal is neither periodic nor common, as is the reversal of heart beat in ascidians and Lepidoptera (Gerould, 1929). Rather, it may be considered as a disturbance such as that reported by Bremer (1931) for the reversal of heart beat in operated chick embryos.

The movements of the two lateral walls are seldom exactly synchronous. Usually this difference is small, but cases have been observed in which the walls were completely out of rhythm with each other. Usually the beats alternate in such cases, although one lateral wall may be motionless while the other behaves normally. In such cases the embryos do not appear retarded in their development. Moreover, the typical manner of contraction is eventually regained. Unilateral beating does not tend to cause the embryo to rotate in the egg, as might be suggested for the cause of the rotation of the embryo around the egg after blastokinesis. In one case of three days of one-sided contraction, which later returned to normal, no rotation was detected.

Several cases have been recorded of double systoles. In these the lateral walls contract normally; but as they commence to relax, a second contraction follows "on the heels" of the first. After this has been spent, the walls relax and remain in diastole until the initiation of the next contraction. The second impulse is usually much weaker than the first, as is evidenced by the fact that it often dies out before reaching the anterior end of the walls.

Embryos whose lateral walls are beating much more rapidly or more slowly than the average for corresponding stages seldom complete development.

The lower increment of contraction-rate increase after yolk engulfment (Figs. 1 and 2) is of special interest, since at this stage a dip in the oxygen consumption curve was found by Boell (1935) in his study of the respiration of *Melanoplus differentialis* embryos. It is interesting that this correlation exists between oxygen consumption and rhythmic movements, although Walker (1935) found that different oxygen tensions did not directly influence the rate of lateral wall contractions.

The increase in heart rate from its initiation up to hatching has also been observed for cold-blooded vertebrates, as shown by Laurens (1914) for *Ambystoma* and by Anderson (1929) for *Salmo* and *Lacerta*. In chick embryos an increase in heart rate up to about the eleventh day has been found by Cohn (1927), Bogue (1932), and others (see Needham, 1931).

From determinations of the areas under the curves in Figures 1 and 2, it was possible to calculate the total number of contractions performed by an embryo from Stage 1 to hatching. The results are shown in Table I. The total number of contractions appears to be fairly constant in the same species, regardless of the length of time required for development. Thus, a total of some 1.31×10^6 movements were made during the 24 days of incubation of *M. femur-rubrum* at 20° C., while about 1.33×10^6 were made during the 8 days spent by the embryos at 35° C.

The precocious hatching of embryos incubated at high temperatures has also been

found by Gray (1929), who worked on the trout. Elaborating on the idea of the presence of a hatching enzyme, first postulated by Kerr (1900), Gray suggested a higher Q_{10} for the hatching enzyme than for development. This hatching enzyme is supposed to dissolve the membranes inclosing the embryo, thus releasing the young animal without the struggles which are usually described as preceding the breaking of the egg shells. Wintrebert (1912), using the rainbow trout, anesthetized embryos at the hatching stage; and although the movements were abolished, hatching occurred on schedule. Dampf, Hoffmann, and Verela (1925; quoted from Needham, 1931) and Williams (1933) have

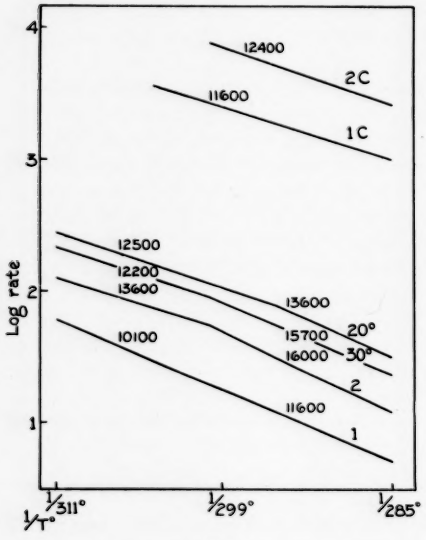

FIG. 7

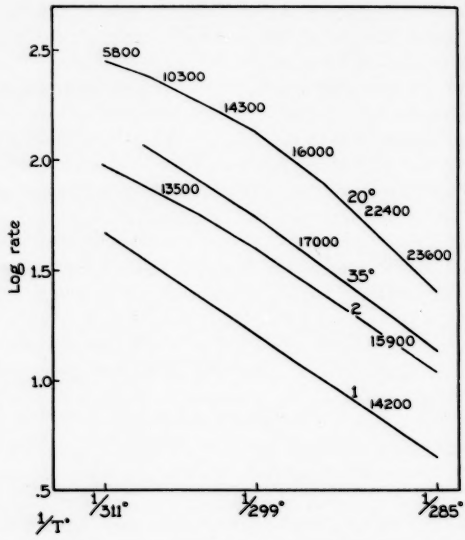

FIG. 8

FIG. 7.—Temperature characteristics for *Melanoplus differentialis*. μ values (determined by calculation) given. 1, embryos at Stage 1. 2, Stage 2. 30°, at hatching, incubated from Stage 1 at 30° C. 20°, at hatching, incubated at 20° C. 1*c*, conductivity of contraction impulse at Stage 1. 2*c*, conductivity of contraction impulse at Stage 2.

FIG. 8.—As in Figure 7 but for *Melanoplus femur-rubrum*.

suggested the presence of hatching enzymes for grasshoppers. However, the rupturing of the cuticle surrounding *Melanoplus* embryos, developed at a constant temperature below 35° C., occurs while the embryo is in movement. The embryo telescopes itself and then literally rams its body against the cuticle. After elongating, the back is arched; and the region between the head and pronotum seems to swell, pressing against the cuticle. It is in this region that the first split in the cuticle invariably appears. It would seem that hatching is brought about by a weakening of the cuticle, which, however, must be finally broken by the body movements of the embryo.

Crozier (1926) found that the temperature characteristics of heart rates were featured by a fair constancy with thermal changes, and that the value for μ was around 16,000.

The values obtained for the rhythmic contractions of grasshopper embryos correspond fairly well with this number (see Fig. 7 and 8).

Crozier (1926) considers the temperature characteristic to be indicative of the "master reaction" of a catenary series of reactions underlying the process studied, a concept derived from the Blackman (1905) principle. "Breaks" in the log rate/(1/T) curve are considered as points at which another reaction becomes the "master"; and a different characteristic value is obtained since each reaction is supposed to have its own constant-temperature characteristic. This idea has been criticized by Heilbrunn (1925) and Belehradek (1931). Buchanan and Fulmer (1930) have shown that suitable choice of abscissa and ordinate scales alter the straight lines between "critical temperatures" (at which "master reactions" change) to a curve. Attention is called to the curve for *M. femur-rubrum* eggs kept at 20° C. to the time of hatching, and then observations made on the effect of temperature changes on the heart rate. A different μ-value for each temperature interval was found. It is rather difficult to suppose a new "master reaction" appearing at each temperature.

The μ for the rate of conduction of the contraction impulse is identical with the characteristic for the rate of contraction at the same stage. Thus, at Stage 1, μ for both conduction and contraction rate is 11,600 for the temperature range 16°–20° C., and at Stage 2 the characteristic for both is 12,400.

When placed at the same temperature, the embryos which had developed at 20° C. were found to have a much more rapid heart beat than those at 30° C. (*M. differentialis*) or 35° C. (*M. femur-rubrum*) (Figs. 3 and 4). Heart rates of embryos incubated at 25° C. were also determined (at the time of hatching) over the same temperature range and were found to be intermediate between the results obtained for 20° C. and 30° C. (or 35° C.) eggs. Since the 30° C. and 35° C. embryos hatched at earlier developmental stages than embryos incubated at lower temperatures, it might be thought that the rate of heart beat for 35° C. embryos at their hatching time would not be comparable to the rate at hatching of embryos which had developed more slowly. The heart-rate/temperature curve of 20° C. embryos (*M. femur-rubrum*) at 20 days is higher than that of the 35° C. group at hatching. The morphological development, as far as pigmentation is concerned, is identical for the two groups at that time.

SUMMARY

1. Lateral wall and heart contractions of *Melanoplus differentialis* and *M. femur-rubrum* embryos were studied at different constant temperatures from the post-blastokinesis stage to hatching. The movements increase slowly until yolk engulfment is complete. A short period of little or no increase is followed by a rapid increase up to or shortly before hatching.

2. The behavior of the two species was similar as regards rate of rhythmic movements. They differ in that *M. femur-rubrum* has a higher thermal developmental range and requires less time for hatching from blastokinesis.

3. The rhythmic lateral body-wall movements become resolved into those of the heart contractions, as Nelsen (1931) suggested.

4. Temperature characteristic values for the lateral wall and heart movements correspond with those found by Crozier as general for heart movements (about 16,000).

5. The rate of conduction of the contraction impulse was low at first but rapidly increased. Its μ is identical with that of the contraction rate at the same stage.

6. Hearts of embryos incubated up to the time of hatching at lower temperatures beat at faster rates than those incubated at higher temperatures, if both are brought to the same temperature.

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GASEOUS METABOLISM IN INJURY AND DEATH OF A CLADOCERAN

(Three figures)

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IN STUDYING the oxygen consumption of several species of Cladocera, it was observed that the rate of respiration at times suddenly became abnormally rapid. It soon became evident that the abnormally rapid utilization of oxygen was invariably associated with the infliction of an injury to the organism, leading finally to its death. This observation led to the present investigation of the sequence of events in the slow death of the organism when subjected to a number of unfavorable conditions. The work consisted of a study of the effect of KCN, of drying, and of 35° C., a moderately high but injurious temperature for this organism.

MATERIALS AND METHODS

In this work *Daphnia magna* were employed. (When reared at room temperature, the animals become sexually mature and release their first clutch of young in about 6 days after they are freed from the brood pouch of the mother.) The animals were subjected to experimentation immediately after they released their first brood of young. (At this stage in the development the mothers measure about 3 mm. in body length.)

The very minute quantities of oxygen consumed by the organisms were measured by a Thunberg respirometer as modified by Fenn (1927), and later by Obreshkove and Banta (1930), to meet the specific experimental needs in this work. A single individual at a time was utilized, and the gaseous metabolism of the animals was followed after the organisms were subjected to the particular unfavorable condition. Continuous observations of the rate of oxygen consumption were made from the time the animals were placed in the respirometer, through the period of injury, and finally to the death of the organism.

Observations of the rate of oxygen consumption were made each 15 minutes for the first hour after the respirometer reached equilibrium, and thereafter readings were taken each hour. In some cases the experiments were extended for several hours beyond the death-point; and in still other experiments observations on the rate of oxygen consumption were discontinued at various phases during the injury, in order to observe under the microscope the actual state of the organism and to correlate this state with the particular variation in the rate of oxygen consumption. The organisms were considered as dead when the heart and the respiratory appendages showed no motion when removed and examined under the microscope. The organisms were reared at 25° ± 0.1° C. up to the time they were placed in the respirometer. After this the temperature was maintained to ±0.01° C.

OBSERVATIONS AND DISCUSSION

In analyzing the observations, typical experiments are presented here rather than the total data available. Each experiment is treated separately so as to enable us to follow the

course of injury and death of a single individual and to study the relation of this course to the gaseous metabolism of the animal. In Figure 1 are shown five typical experiments of the results obtained of the effect of 35° C., a temperature injurious to *D. magna*. The percentage of increase and decrease in the rate of oxygen consumption per individual per hour, as compared with the average rate of respiration during the first hour at this temperature, is plotted as a function of the time the organism was subjected to the unfavorable condition. The time curves of the process of injury and death and of their relation to gaseous metabolism of the organism reveal striking general results common to all

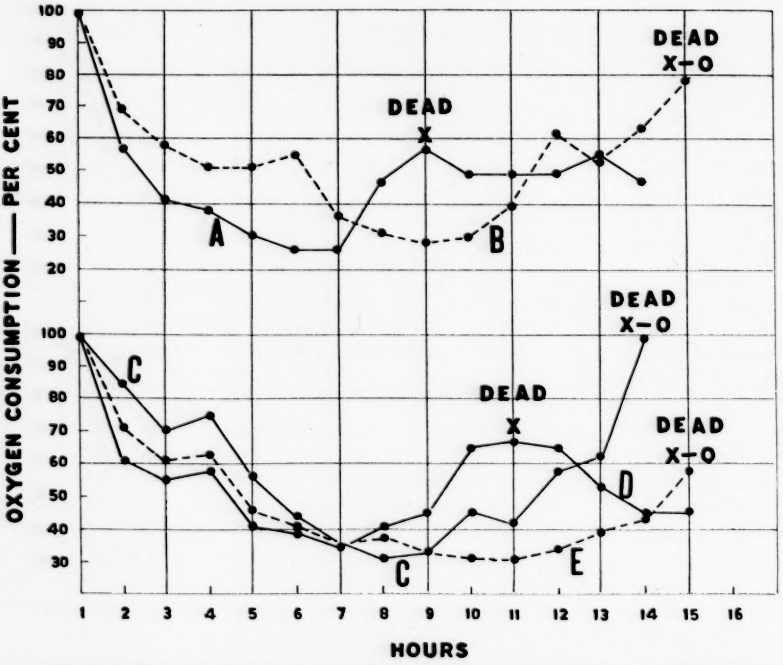

FIG. 1.—Typical effects of 35° C. on the rate of oxygen consumption of *D. magna*. The percentage of increase and decrease in this rate per individual per hour is plotted as a function of the time the organism was subjected to the unfavorable condition. The point marked *X* in each graphic representation indicates the death-point of the animal, and *X–O* denotes that the particular experiment was discontinued immediately after the death of the animal.

the experiments. From the start the gaseous metabolism of each individual falls rapidly until the rate of oxygen consumption is reduced to a minimum. The maximum degree of respiratory decrease occurs with regularity. The rate of oxygen consumption is seen to fall to about 30 per cent of the initial rate in the course of a period varying from 6 to 11 hours. After this period, and with the approach of death, the rate of oxygen consumption suddenly increases to from 58 to 70 per cent of the normal rate of respiration (Fig. 1, curves *A*, *D*, *E*). In a few cases the rate of oxygen consumption after the characteristic fall rose almost to the normal rate (Fig. 1, curves *C*, *B*), and in still other cases it exceeded this point. The well-defined peak in the graphic representations marked the

death-point of the organism, as determined by sacrificing experiments in various phases of the course of injury.

Just before the initial fall in oxygen consumption is complete, periods of increased rate of respiration of short duration are found in all graphs. In many cases, as may be observed from the graphic representations, these periods set in with surprising regularity, occurring between the third and fourth hour. In two experiments (not given in this paper) these waves of increased rate of respiration reached larger magnitudes.

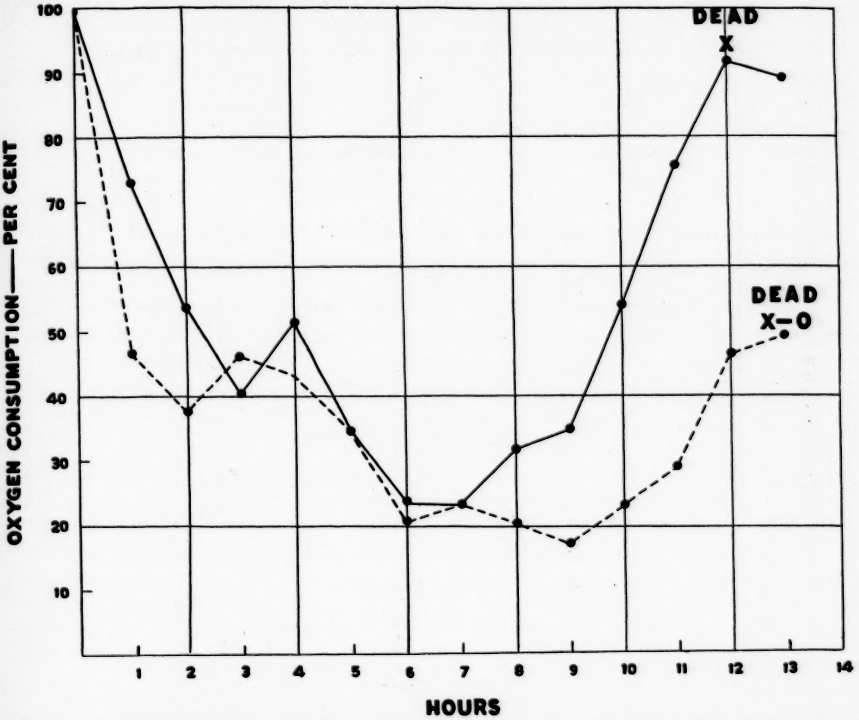

FIG. 2.—Shows typical effects of KCN on the rate of oxygen consumption of *D. magna*. The percentage of increase and decrease in this rate per individual per hour is plotted as a function of the time the organism was subjected to the unfavorable condition. The point marked *X* in each graphic representation indicates the death-point of the animal, and *X–O* denotes that this particular experiment was discontinued immediately after the death of the animal.

Immediately after the death of the organism the rate of oxygen consumption again decreases. It is evident, from inspection of Figure 1, that in some cases even 5 hours after the beginning of this decrease the rate continues its downward course, falling in some cases almost to the initial maximum degree of inhibition in the rate of respiration. The oxygen consumption during this period probably represents tissue respiration. In no case did we study this tissue respiration for more than 5 hours after the death-point.

Daphnia magna subjected to KCN show a remarkably similar sequence of events in the course of injury and death as hereto described pertaining to the influence of injurious

temperature. The experiments with KCN were fewer in number. For the effect of N/10,000 KCN at 25° C., the rate of oxygen consumption under this condition fell to about 20 per cent of the normal rate at the same temperature. The whole metabolic sequence, consisting of the initial decrease in the rate of oxygen consumption, the increase with the approach of death, and the peak of this wave followed by a fall in the gaseous metabolism, is again in evidence (Fig. 2).

A study of the gaseous metabolism in injury and death was also made when *D. magna* were subjected to drying. In drying, the progressive changes produced are clearly the

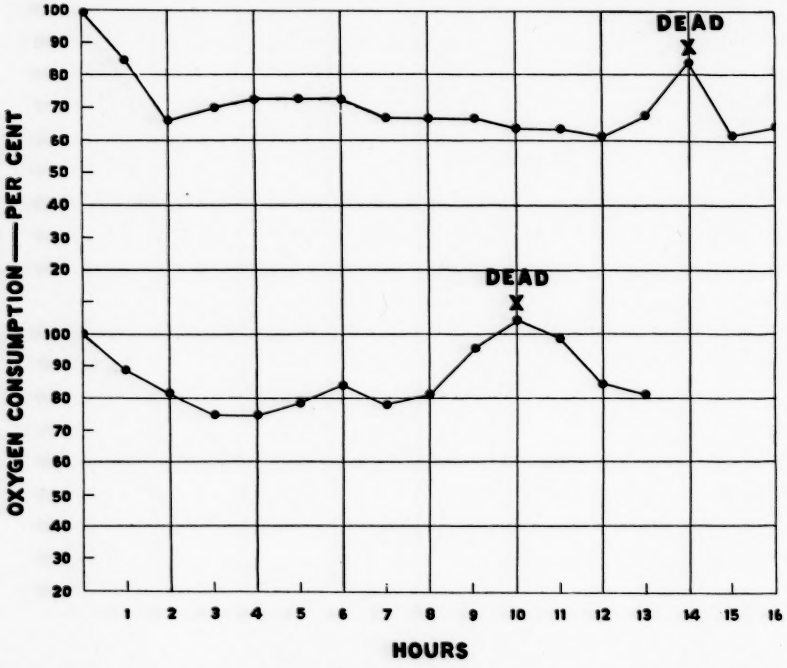

FIG. 3.—Shows typical effects of drying on the rate of oxygen consumption of *D. magna*. The percentage of increase and decrease in this rate per individual per hour is plotted as a function of the time the organism was subjected to the unfavorable condition. The point marked *X* in each graphic representation indicates the death-point of the animal.

results of the disturbances of the respiratory mechanism. The individual which was to be subjected to experimentation was dried with filter paper, and only a very thin film of fluid was left between the two flaps of the carapace. The experimental conditions thus induced are of considerable interest in view of the fact that *D. magna* is strictly an aquatic animal. Animals thus treated were able to remain alive in the majority of cases from 10 to 14 hours, but in some cases this period was extended beyond 20 hours. Typical death curves and the time relations involved are given in Figure 3. A comparison of these graphic representations with each other and with those produced under the other unfavorable conditions described reveals that the general features of the injury

and death curves are the same, with the exception that, in drying, the drop in the oxygen consumption rate to the minimum value so characteristic of all experiments exhibited a higher level. Drying reduces the oxygen consumption to about 70 per cent of the normal respiration. Moderately high temperature and N/10,000 KCN, on the other hand, reduce the gaseous metabolism approximately to 30 per cent and 20 per cent, respectively, before the characteristic rise leading to the death of the organism was observed.

The fact that in each case in the course of injury and death the gaseous metabolism first fell and then rose suddenly and sharply is of especial interest. Osterhout (1922), in studying the rate of carbon dioxide excretion of *Laminaria* when subjected to various toxic substances, has observed only a gradual decrease in this rate with the approach of death. It would, therefore, be interesting to know whether the phenomenon here reported is a characteristic of the organism as a whole, indicating a sudden change in the respiratory mechanism, or whether similar effects could be obtained with isolated tissues.

In the experiments herein reported the animals subjected to experimentation were not bacteria-free when placed in the respirometers. In the Thunberg respirometers used, however, an equivalent volume of the medium surrounding the organism is introduced into a balancing-cup; and the bacterial respiration would, consequently, be balanced, giving no net effect on the readings. It is, moreover, inconceivable to the authors that the rate of bacterial respiration would decline in the cup containing the organism subsequent to its death. It is therefore believed that the trend of the gaseous metabolism of *D. magna* reported in this paper is a true accompaniment of the injury and death of the organism under these circumstances, although it may be somewhat complicated by bacterial action.

SUMMARY

1. The changes in the gaseous metabolism of *D. magna* were observed in injury and death, after the organisms had been subjected to unfavorable conditions.

2. The work consisted of a study of the effect of KCN, of drying, and of 35° C., which is a moderately high temperature for this organism.

3. Continuous observations made under each of these conditions furnished data of interest concerning the sequence of events in the slow death of the cells and of the organism as a whole.

4. An early upset was observed in the normal balance of the organism. From the start the gaseous metabolism of the individuals decreased rapidly until the rate of oxygen consumption was reduced finally to a minimum.

5. With the approach of death, there was observed an increase in the rate of oxygen consumption.

6. The highest point in the rate of oxygen consumption in the graphic representations marked the death-point of the animal.

7. After the death of the organism, again a decrease was observed in the rate of oxygen consumption. The oxygen consumption during this period undoubtedly represents tissue respiration.

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NUTRITION STUDIES WITH THE COCKROACH (BLATTELLA GERMANICA)

(One figure)

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THE common cockroach, *Blattella germanica*, was selected for a nutritional study of a generalized insect because it has been used for numerous morphological studies which are an adequate basis for physiological work. The omnivorous feeding habits, incomplete metamorphosis, fast growth-rate, and rapid reproduction make the roach well suited for this type of experimental work.

A stock colony of cockroaches was maintained in order to have available experimental animals of known nutritional history. Fish aquaria, tightly covered with several layers of cheesecloth, served as adequate containers for rearing stock. Our diet for stock rats (Maynard, 1930), or a mixture of equal parts of dried skim milk and ground whole wheat, provided a satisfactory diet for growth and reproduction. An experimental container utilizing a half-pint milk bottle was developed in this laboratory (McCay and Melampy, 1936), a photograph of which is shown in Figure 1. The water supply was provided by a wet cotton plug in a vial fastened by means of a cork to a perforated milk cap. The diets were placed in a small paper cup on the bottom of the bottle.

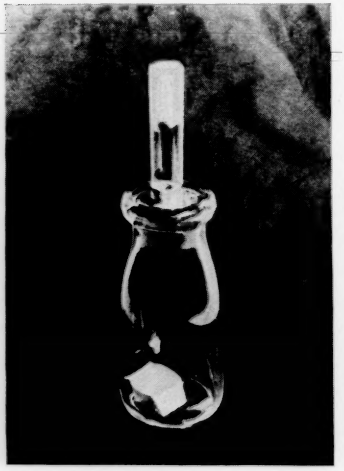

FIG. 1.—Photograph of experimental container.

Preliminary experiments were run in order to study the effect of ethyl ether as an anesthetic and to ascertain the suitability of the container. The insects were of the same approximate weight in the beginning. These experiments were conducted at a temperature of 22°–24° C. The duration of the experiment was 82 days. Group I received their food in elevated, side-arm food containers and were anesthetized and weighed every 14 days. The average final individual weight attained was 76 mg. In Group II the insects received their food in small containers on the bottom of the cage and were anesthetized every 14 days. These insects weighed 80.8 mg. at the close of the experiment. Group III received their food on the bottom of the cage and were treated with ether only at the beginning of the experiments. The group attained the final average weight of 77 mg. These experiments demonstrated that under the conditions of the experiments there is no effect on the increase in mass of the cockroach by the use of ethyl ether as an anesthetic and that the position of the food does not retard the growth.

NATURAL FOODS AS DIETS FOR THE COCKROACH

Females with well-developed capsules were placed in experimental containers in order that the date of emergence of the young insects could be ascertained. The insects used in experiments were so selected, when 7 days old, that the average weight was 2.5–3.0 mg. One hundred and fifty to three hundred insects were used for each experiment. The insects were kept in a dark, constant-temperature room at 27°–28° C. The roaches were anesthetized with ethyl ether and weighed in a tared weighing bottle on an analytical balance, and were placed in groups of ten in experimental containers. The weight attained in 42 days was used as a measure of the physiological efficiency of the diets fed. This period of time was selected in order to eliminate metabolic changes which accompany the reproductive processes.

The first experiment was designed to study the physiological response of the developing cockroach to various natural foodstuffs. The diets and results are shown in Table I.

TABLE I

COMPARATIVE NUTRITIONAL VALUE OF NATURAL FOODS FOR THE DEVELOPING COCKROACH

Diet No.	Dietary Ingredients	Average Final Weight (Mg.)
1	Dried whole milk	6.3
2	Dried skim milk	10.9
3	Commercial baby food	4.4
4	Ground whole wheat	19.1
5	Bolted wheat flour	4.1
6	1:1, whole wheat and dried skim milk	22.8
7	1:1, whole wheat and dried blood	22.3
8	Dried beefsteak	23.5

From the data in this table we conclude that a mixture of equal parts of ground whole wheat and dried skim milk makes a satisfactory diet for the growing cockroach. The differences between whole wheat (diet No. 4) and bolted wheat flour (diet No. 5) are similar to those reported by Sweetman and Palmer (1928) in their work with *Tribolium confusum*, showing that the larval period was shorter in ground whole wheat than in patent flour. The comparatively poor results obtained with dried whole milk and the commercial baby food clearly demonstrates that the requirements for the developing roach are quite different from those of the young mammal.

THE REQUIREMENT OF THE COCKROACH FOR AN ETHER-SOLUBLE SUBSTANCE

In a second experiment, diet No. 6 was selected as a basal diet for the study of lipid metabolism of the cockroach. This diet was dried *in vacuo* and extracted in a Soxhlet extractor for 24 hours with anhydrous ethyl ether in order to provide a fat-free diet for comparative study and for use as a basal diet with specific fats. A purified diet (No. 10) was made of cooked starch, purified casein and lactalbumin, and Harris vitamin B concentrate. This diet was used to assay the growth-promoting value of the ether extract of diet No. 6. The cooked starch was prepared by heating raw starch with steam and later drying and pulverizing it. Commercial casein and lactalbumin were made free

from vitamins A and B by washing twice daily for one week with 0.1 per cent acetic acid, followed by three extractions with 60 per cent alcohol and one with 95 per cent alcohol, and finally washing with ethyl ether. The material was then spread in pans and dried in the air. The diets used in the second experiment and the results obtained are given in Table II.

The data in Table II show that anhydrous ethyl-ether extraction of a satisfactory diet removes a substance necessary for the development of the cockroach and that this extract improved a purified, low-fat diet. Further investigation was made concerning the growth-stimulating substance, using ground whole wheat (diet No. 5) as the basal diet in place of No. 6. The reason for doing this was that roaches grew fairly well on wheat alone and that it was a simple diet in that it contained only one ingredient. When

TABLE II

ETHYL-ETHER-SOLUBLE, GROWTH-PROMOTING SUBSTANCE OF A WHEAT-MILK DIET

Diet No.	Dietary Ingredients	Average Initial Weight (Mg.)	Average Final Weight (Mg.)
6	Ground whole wheat and dried skim milk	2.94 ± 0.08	32.97 ± 1.49
9	Ground whole wheat and dried skim milk. Dried *in vacuo*. Extracted with ethyl ether for 24 hours	2.94 ± 0.08	11.8 ± 0.68
10	79 gm. cooked starch 15 gm. purified casein 5 gm. purified lactalbumin 1 gm. vitamin B concentrate	2.72 ± 0.06	12.3 ± 0.97
11	100 gm. of diet No. 10 plus ethyl-ether extract of 100 gm. of diet No. 6	2.61 ± 0.08	23.31 ± 1.20

dried ground whole wheat was subjected to anhydrous ethyl-ether extractions, the results obtained with it were practically identical with those of diet No. 9. Thus evidence was furnished that the growth-promoting substance was an ethyl-ether-soluble constituent of ground whole wheat.

In order to ascertain whether the extraction process itself may destroy some of the growth-promoting activity of the whole wheat, 100 gm. of ground whole wheat were dried *in vacuo* at 65° C., extracted for 24 hours with anhydrous ethyl ether, and the extract added to the residue to form diet No. 12. The insects did as well on this diet as on the original ground wheat, as is shown in Table III.

Because of the yellow color of ethyl-ether extract of wheat it was suggested that the extraction may remove carotinoid pigments essential for the development of the roach. On spectrographic examination there was general absorption in the blue and violet range characteristic of the carotinoid pigments. However, the addition of 3 per cent butter oil of a known vitamin A potency to the extracted wheat did not improve its nutritional value (diet No. 14, Table III).

During the last decade experimental evidence has been presented showing that specific fats may be concerned in the physiology of animals. Evans (1928) has demonstrated

that the fat-soluble vitamin E is related to growth and vigor as well as reproduction. Burr and Burr (1930) have produced a deficiency disease in rats by the rigid exclusion of fats from the diet. A small amount of lard daily had a curative effect. In the light of this work a diet of ether-extracted wheat with 3 per cent lard was fed a group of insects; but this fat did not substitute for the ether extract of wheat, as is shown by the data for diet No. 15 in Table III.

By the use of a highly purified basal diet Blumberg (1935) was able to demonstrate a growth-deficiency disease in rats curable by wheat oil. In distribution and general properties, the growth principle resembled vitamin E inasmuch as the growth factor withstood saponification and was concentrated in the unsaponifiable fraction. To test this for insects, about 4.5–5.0 gm. of the ether extract of wheat were saponified for 6

TABLE III

COMPARISON OF FAT SUPPLEMENTS TO A DIET OF ETHER-EXTRACTED WHEAT

Diet No.	Dietary Ingredients	Average Initial Weight (Mg.)	Average Final Weight (Mg.)
5	Ground whole wheat dried *in vacuo*	2.6 ± 0.0	25.6 ± 0.04
12	Diet No. 5 extracted with anhydrous ethyl ether for 24 hours	2.63 ± 0.003	11.4 ± 0.047
13	Diet No. 12 extract replaced on residue	2.80 ± 0.003	26.6 ± 0.07
14	Diet No. 12 plus 3 per cent butter oil	2.49 ± 0.002	12.4 ± 0.003
15	Diet No. 12 plus 3 per cent lard	2.63 ± 0.003	11.0 ± 0.002
16	50 gm. of diet No. 12 plus saponifiable fraction of 4.5 gm. of wheat extract	2.85 ± 0.003	24.8 ± 0.084
17	50 gm. of diet No. 12 plus unsaponifiable fraction of 4.5 gm. of wheat extract	2.99 ± 0.003	27.8 ± 0.089

hours on a steam bath by the use of 20 per cent alcoholic KOH. The saponifiable and unsaponifiable fractions were replaced on extracted wheat and fed. The diets (Nos. 16 and 17) and the results obtained are shown in Table III.

We conclude from the data presented in Table III that anhydrous ethyl ether removed a substance from whole wheat which is not replaceable either by lard or butter oil. The ethyl-ether extract of wheat retains its growth-stimulating characteristic after saponification, and the greater activity is in the non-saponifiable fraction. In this respect the growth factor is similar to that reported by Hobson (1935) for *Lucilia*. Moskalenko (1933) found wheat germ oil the most effective for stimulating the growth of cockroaches being fed a purified diet. Bacot and Harden (1922) reported that an 80 per cent alcoholic extract of wheat germ was effective for the development of *Drosophila*. Sweetman and Palmer (1928) obtained better results with *Tribolium* when fat was present in the diet. According to Richardson (1926), extracted whole-wheat flour was unsatisfactory for the development of *Ephestia*.

STUDIES OF SOURCES OF THE VITAMIN B COMPLEX

As was pointed out earlier, the purpose of this work was to develop a diet which would enable us to study the lipid metabolism of the cockroach. Previous attempts, as described, were made to reduce the fat content of the natural diet, with the result that the food was unsatisfactory for the developing roach. In order to study the problem, it was necessary to devise a low-fat, purified diet which would permit satisfactory development of the roach. Our experiments were continued by the use of diets composed of lactalbumin, skim-milk ash, Harris vitamin B concentrate, and butter oil. The proteins and carbohydrate materials were pulverized in a ball mill. The fat was added by means of an ether solution, and later the solvent was removed by heating on a steam bath. The diets and results are shown in Table IV. The average final weight attained by the insects

TABLE IV

PURIFIED DIETS WITH DIFFERENT LEVELS OF VITAMIN B CONCENTRATE

Diet No.	Lactalbumin (Gm.)	Skim-Milk Ash (Gm.)	Butter Oil (Gm.)	Sucrose (Gm.)	Vitamin B Concentrate (Gm.)	Average Final Weight of Insects (Mg.)
22	20	3.5	5	71.5	0	7.95 ± 0.28
23	20	3.5	5	70.5	1	15.14 ± 0.61
24	20	3.5	5	69.5	2	19.5 ± 0.07
25	20	3.5	5	68.5	3	28.2 ± 1.23
26	20	3.5	5	67.5	4	28.7 ± 1.43
27	20	3.5	5	66.5	5	27.8 ± 1.97

TABLE V

PURIFIED DIETS WITH DIFFERENT LEVELS OF DRIED YEAST

Diet No.	Purified Casein (Gm.)	Purified Lactalbumin (Gm.)	Cooked Starch (Gm.)	Dried Bakers' Yeast (Gm.)	Average Initial Weight (Mg.)	Average Final Weight (Mg.)
28	20	10	70	0	3.28 ± 0.13	6.99 ± 0.31
29	20	10	70	1	3.52 ± 0.09	8.14 ± 0.36
30	20	10	70	5	2.54 ± 0.55	15.30 ± 0.87
31	20	10	70	10	2.89 ± 0.65	22.35 ± 0.78
32	20	10	70	25	2.41 ± 0.001	45.5 ± 0.05

was a linear function of the amount of vitamin B concentrate fed up to the two highest levels, where a plateau of development was reached. The roaches did not develop as well as those receiving diet No. 6 (Table II), and thus it was concluded that this series of purified diets would not permit satisfactory development for our purposes.

Another series of experiments was carried out in which a combination of casein and lactalbumin was used as a protein source and cooked starch as carbohydrate. The vitamin B complex was supplied by dried bakers' yeast as purchased from the Northwestern Yeast Company, Chicago. The diets and results are shown in Table V. With increasing amounts of dried yeast there was a corresponding increase in the final weight attained by the insects. Diet No. 32 fulfilled our requirements of a low-fat diet suitable for metabolic work with the cockroach.

While conducting experiments on the development of a suitable purified diet, the heat stability of the vitamin B complex of the dried yeast was investigated. Diet No. 33 was prepared identical to diet No. 32 with the exception that the yeast in the former had been heated for 144 hours at 105° C. Elvehjem (1933) reported that under these conditions vitamin G is inactivated. The growing roach developed as well on this diet as on diet No. 32 (Table VI). The yeast used in diet No. 34 (Table VI) had been autoclaved at 120° C. for 6 hours at a pressure of 15–18 pounds. The insects receiving it attained a final weight of 77 per cent of those receiving the untreated yeast in diet No. 32, thus showing the destructive effect of the higher temperature. Autoclaving is the common method of inactivating vitamin B in yeast. Hobson (1933) reported that autoclaving yeast at an alkaline pH did not destroy the growth-promoting properties of the dried yeast for *Lucilia*. This finding was tried and confirmed by us. The yeast was auto-

TABLE VI

HEAT STABILITY OF THE VITAMIN B COMPLEX OF DRIED YEAST

Basic diet No. 32 was used in each test.

Diet No.	Yeast Treatment	Average Initial Weight (Mg.)	Average Final Weight (Mg.)
32	None	2.41 ± 0.001	45.5 ± 0.05
33	Heated 144 hours at 105° C.	2.64 ± 0.00	45.3 ± 0.06
34	Autoclaved 6 hours at 120° C.	2.69 ± 0.002	35.4 ± 0.08
35	Autoclaved 3 hours at 120° C. in N/20 NaOH	2.93 ± 0.004	43.6 ± 0.078

claved in a N/20 solution of NaOH. The mixture was autoclaved at 120° C. at 15–18 pounds pressure for 3 hours. After this treatment the digest was made neutral to litmus with HCl. The material was dried and used in diet No. 35 (Table VI). From these experiments we concluded that the vitamin B complex requirements of *B. germanica* are different from those of higher animals but similar to those reported for other insects.

A STUDY OF DIETS CONTAINING VARYING AMOUNTS OF SUCROSE AND FAT

For a study of the lipid metabolism of the cockroach it is desirable to have a diet as low as possible in ether extract so that the physiological effect of various kinds of fat can be studied. Diet No. 32 (Table V) was satisfactory in all respects as far as growth was concerned, but the carbohydrate consisted of starch which contains a variable amount of fat that is difficult to remove. Thus an experiment was set up in which sucrose was used in place of starch and in which the effects of varying fat levels were observed. The diets and results are shown in Table VII. The diet designed to be as low in fat as possible (diet No. 41) contained 0.39 per cent of ether extract.

FACTORS INFLUENCING FAT DEPOSITION IN THE COCKROACH

A group of experiments was designed to study the influence of the quality of dietary fat on the body lipids, by the use of fats of different degrees of saturation. The results,

presented in Table VIII, demonstrate that the degree of saturation of the body lipid can be varied by feeding fats of different iodine numbers. The alimentary tract was removed from all insects so that undigested food would not invalidate the chemical analysis. The preparation of the tissue and the method of analysis was that of Bloor (1928) and Yasuda (1932). The final percentages of total lipid were calculated on the gut-free, fresh tissue.

Kellner (1886) suggested that silkworms form fat from protein during their larval period. Weinland (1908) presented data based on experiments with *Calliphora* showing that these insects were able to synthesize fat from protein substances such as Witte's

TABLE VII

THE INFLUENCE OF VARYING AMOUNTS OF CARBOHYDRATE AND FAT IN THE DIET

Diet No.	Purified Casein (Gm.)	Purified Lactal-bumin (Gm.)	Sucrose (Gm.)	Butter Oil (Gm.)	Lard (Gm.)	Bakers' Yeast (Gm.)	Average Initial Weight (Mg.)	Average Final Weight (Mg.)
41	20	10	70	0	.....	25	2.56 ± 0.002	26.1 ± 0.055
42	20	10	60	10	.....	25	2.65 ± 0.001	30.7 ± 0.027
43	20	10	50	20	.....	25	2.91 ± 0.00	39.9 ± 0.039
44	20	10	0	70	.....	25	2.79 ± 0.002	18.3 ± 0.114
47	20	10	0	0	70	25	2.78 ± 0.008	16.2 ± 0.45

TABLE VIII

THE INFLUENCE OF DIETARY FATS OF DIFFERENT DEGREES OF SATURATION ON THE TISSUE LIPIDS OF THE ROACH

Diet No.	Iodine No. of Dietary Fat	Fat in Diet (Per Cent)	Total Lipid in Fresh Tissue (Per Cent)	Iodine No. of Tissue Lipid
46*	7	28	7.8	51
44 (see Table VII)	27	56	21.0	59
47 (see Table VII)	49	56	13.2	64

* 35 gm. coconut oil, 35 gm. sucrose, 20 gm. purified casein, 10 gm. purified lactalbumin, and 25 gm. dried bakers' yeast.

peptone. Bacteria were not concerned, but the enzymes responsible for the process came from larval tissue. The fat-synthesizing character was found only in the larvae, not in the eggs. Hofmann (1872) was the first to prove that insects are able to build fats from protein by feeding *Musca domestica* on defibrinated blood and determining the fat content of the food, the eggs of the flies, and their larvae. Dumas and Milne-Edwards (1844) reported experiments in which bees fed honey exclusively produced individually as much as 0.0106 gm. of wax. Lusk (1928) has shown that proteins can be used by higher animals for fat synthesis, but the processes are more rapid when carbohydrates and fats are available.

An experiment was run to study the response of the roach to diet in which the carbo-

hydrate had been replaced by a mixture of the proteins casein and lactalbumin. The only lipid present was that contained in the yeast. The insects attained an average final weight of 26.4 ± 0.057 mg. The total lipid content was 1.90 per cent, and the iodine number was 69. This experiment demonstrates that the roach can develop on a high protein diet but that the lipid is reduced and the iodine number is increased.

Roaches were selected from the stock colony in order to study the effect of sex differences and degree of maturity on body fats. These insects had received diet No. 6 (Table I). The data presented in Table IX show that the females contain nearly two and one-half times as much total lipid as the males.

TABLE IX

THE EFFECT OF AGE AND SIZE ON THE BODY LIPIDS OF *Blattella germanica*

	Percentage of Total Lipid	Iodine No.
Males	1.70	74
Females with egg capsules	4.78	74
Nymphs	5.72	69

Two groups of mature males were selected from the stock colony for experiments to study the effect of inanition on the roach. One lot received food throughout the experimental period, and the other group received nothing but water. When 50 per cent of the latter group had died from starvation, the remainder were killed, dissected, and analyzed for total lipid. The average original weight for both groups was 60 mg., and the average final weight of the starved insects was 51.5 mg. Those on the control diet showed little or no weight loss. The iodine number of the starved animals was lower, but there was little difference in the total lipid content of the two series of insects.

SUMMARY

Diets composed of milk and its products which permit a young mammal to develop were unsatisfactory for the growing roach. This insect responded equally well to a diet of ground wheat and dried skim milk and to one of the same cereal with meat products.

The ether extract of wheat furnished a growth-stimulating substance which withstood saponification, and the unsaponifiable fraction possessed the greater activity. Neither lard nor butter oil could be substituted for the wheat extract.

The vitamin B complex of dried yeast required by *B. germanica* was heat- and alkali-stable.

A purified diet of a low fat content was developed which permitted satisfactory development of the growing cockroach.

Blattella germanica was able to convert protein into body lipid. The nymphs had the largest amounts of body fat, while the females possessed two and one-half times as much lipid as the males. In starvation the amount of fat was not greatly reduced, although the iodine number was diminished. The degree of saturation of tissue fat was altered by feeding fats of different iodine numbers.

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STUDIES ON THE FIREFLY. I. THE EFFECTS OF LIGHT AND OTHER AGENTS ON FLASHING IN PHOTINUS PYRALIS, WITH SPECIAL REFERENCE TO PERIODICITY AND DIURNAL RHYTHM[1]

(Nine figures)

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IT IS well known that in nature the males of the firefly *Photinus pyralis* ordinarily remain quiescent during the bright light of day and do not flash; that in the dim light of dusk, at about 7:00 P.M., they become active and fly about and flash; and that at about 9:00 P.M. with the onset of darkness, they become quiescent again and remain so until the next evening.

Mast (1912) discussed in detail the time of appearance ("first glow") of *P. pyralis* in the evening. He observed that "the time of the day the first glow appeared was surprisingly constant"; and he concluded, "It appears that the activity of these creatures is a periodic phenomenon to a considerable extent independent of immediate environmental changes."

Allard (1931) made extensive observations on the same species and concluded that flashing begins earlier on cloudy than on clear days, and that the time at which flashing begins gradually becomes earlier as the season progresses.

Rau (1932) maintained that in the darkest and lightest areas near his laboratory there was a consistent difference of from 25 to 40 minutes in the time at which the first flash occurred in this species. He also observed on one afternoon that the fireflies flashed when it became very dark just before a thunderstorm.[2] He concluded that "these creatures are stimulated to action by definite light conditions and not by a blind rhythmic impulse from within."

Mast noted that "low temperature . . . . seems to retard their appearance." Allard made a large number of records of the temperature at the time of the first appearance of the fireflies in the evening but did not draw any conclusion.

There are, then, two antagonistic views with reference to the influence of light on flashing in the firefly. Mast, on the one hand, is inclined to minimize the effects of light and to emphasize internal factors; whereas Allard and Rau, particularly the latter, hold that light intensity and time of appearance are closely related and that internal factors are not involved.

The purpose of this investigation is to clarify the disputed views presented above and to ascertain more precisely the relations between various environmental factors (particularly light) and flashing in the firefly. The results presented were obtained entirely from work done on males of the firefly *Photinus pyralis* Linn. at Baltimore, Md.

[1] This work represents part of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Johns Hopkins University. It was performed under the direction of Professor S. O. Mast, whose generous advice and assistance the author is happy to acknowledge.

[2] Observed also by Laurent (*Entomological News*, **25** [1914], 334) and by the present writer.

OBSERVATIONS IN THE FIELD

The writer attempted to put the field observations on a more accurate basis by measuring, on a considerable number of evenings, both the light and the temperature at the time and the place of the first flash observed. The main results of these observations are presented in Table I. This table shows that all of the factors recorded varied somewhat, but always within fairly narrow limits. Comparison of observation 6 with 7, and 15 with

TABLE I

RELATION BETWEEN TEMPERATURE, LIGHT INTENSITY, AND THE TIME OF FIRST FLASH

The observations are arranged in the order of increasing light intensity. The lines drawn through the fourth column separate groups of light intensities which were averaged together and plotted against the averages of the corresponding temperatures (col. 5) in Figure 1.

Observation	Date (July, 1933)	Weather	Light Intensity (Meter-Candles)	Temperature (Degrees Centigrade)	Time of First Flash (P.M., E.S.T.)
1	27	Hazy	26	21.1	7:15
2	19	Hazy	35	23.9	7:10
3	14	Cloudy	40	17.8	7:28
4	11	Hazy	41	22.0	7:20
5	28	Cloudy	45	22.3	7:01
6	5	Clear	55	23.3	7:22
7	10	Overcast	55	23.3	6:40
8	7	Clear	55	20.5	7:24
9	16	Clear	56	25.5	7:08
10	13	Hazy	70	21.7	7:10
11	20	Hazy	75	26.7	6:58
12	17	Hazy	79	25.0	7:08
13	25	Overcast	84	26.7	6:59
14	18	Hazy	87	24.4	7:06
15	12	Clear	100	22.3	7:06
16	15	Overcast	100	22.3	6:45
17	6	Clear	100	25.5	7:16
18	21	Clear	110	26.7	7:10
19	24	Hazy	130	25.5	7:01
20	8	Cloudy	140	26.7	7:10

16, indicates that (other conditions being equal) the fireflies tend to begin to flash earlier on cloudy than on clear days, i.e., that they respond to light of a specific intensity. Comparison of observation 8 with 9, and 10 with 11, indicates that at a given light intensity the fireflies begin to flash earlier in high than in low temperatures.

Figure 1 shows the results obtained if the light intensities recorded in Table I are grouped according to the dividing lines in the fourth column and the light intensities in each group are averaged together and plotted against the averages of the temperatures corresponding to each group, i.e., the light intensity at the time and place of first flash is plotted against the temperature at the same time and place. This figure shows that

temperature and light are interrelated factors in their effect on the time of the first flash, and that, for each degree of rise in temperature, flashing begins at a light intensity about 20 meter-candles more intense. Figure 1 indicates, in addition, that the time at which flashing begins depends primarily upon light and temperature and not upon the hour of day. However, the fact that in nature the fireflies do not flash again at dawn, even though conditions of light and temperature are the same as those under which they flash at dusk, shows that specific light intensity and temperature do not absolutely determine the time of day at which flashing occurs, and that the effect of a given light intensity appears to depend on whether it is reached by decrease from a higher intensity or increase from a lower.

In sum, these results indicate, in accordance with the observations of Allard and of Rau, that the flashing is correlated with light of a definite intensity, and, in accordance

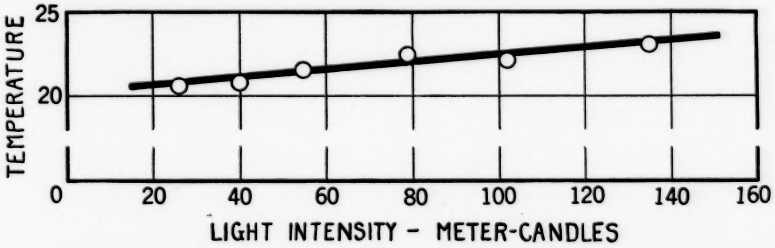

FIG. 1.—Relation between temperature and light intensity at the time of first flash. Graph constructed from the data presented in Table I, by plotting the averages of different groups of temperatures that obtained at the times of the first flashes on different evenings against the averages of the corresponding light intensities. Note the linear relation between temperature and light intensity.

with the observations of Mast, that temperature also has an effect on flashing. However, they offer little in support of the view that flashing is correlated with an inherent periodicity in the organism.

OBSERVATIONS IN THE LABORATORY

APPARATUS AND METHODS

In order to obtain more accurate information concerning the relation between flashing and light intensity than was obtained in observations in the field, an apparatus was constructed in a darkroom, with which it was possible to expose large numbers of fireflies to light of various fairly uniform intensities and to measure accurately the frequency of flashing under different controlled conditions. The apparatus (Fig. 2) consisted essentially of a floodlight (*F*), suspended on pulleys 3 meters above the floor of the darkroom, and a cage (*C*), made of mosquito netting, in which the fireflies were kept during experimentation. The cage was 2.5 m. long, 2 m. wide, and 0.6 m. high. The floodlight consisted of a battery of fifteen 200-watt frosted lamps with tinned 60° conical reflectors (*R*) 25 cm. in basal diameter, fastened to a wooden platform (*P*) 2 m. long and 1 m. wide. Different light intensities were obtained by varying the number of lamps turned on and by placing various cloth screens (*S*), 2×1.2 m., below the lamps on brackets (*B*).

With all the lamps turned on and no screens under them, the intensity of the light was 3,600 meter-candles in the middle of the top of the cage and 1,180 m.c. in the corners at the floor. For the sake of convenience this range of intensity is referred to hereinafter

as 2,500±m.c., which value represents the average intensity of the light in the cage under the conditions described above. With seven alternate lamps turned on and three screens under them, the intensity was 96 m.c. at the top of the cage and 48 m.c. in the corners at the floor. This range is referred to hereinafter as 65±m.c.

The temperature of the darkroom varied between 24° and 25° C.

The procedure in most of the experiments was in general as follows: Males of *P. pyralis* were collected in the field between 7:00 and 8:00 P.M. and put into quart milk bottles, twenty-five in each bottle. A little grass was added to each bottle to maintain favorable humidity. Promptly at 8:00 P.M. the bottles were completely covered with opaque

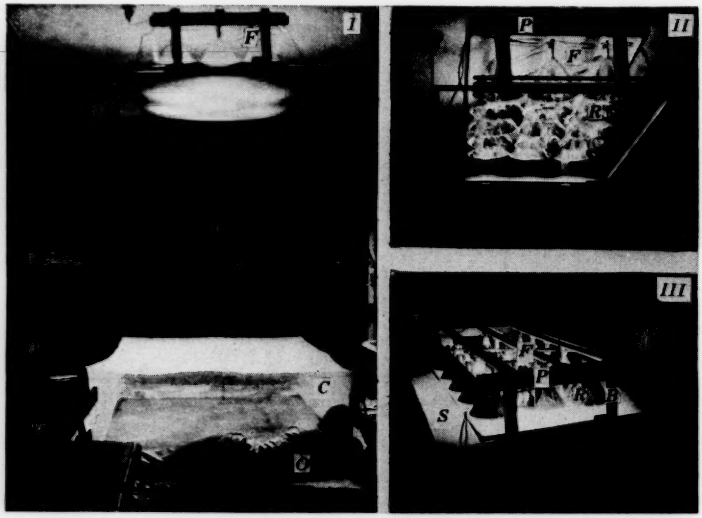

FIG. 2.—Photographs of apparatus used in darkroom to subject fireflies to light of various intensities. *I*, entire apparatus viewed from one end; *II*, lighting unit viewed from below; *III*, lighting unit viewed from above; *F*, floodlight; *C*, cage made of mosquito netting (2.5×2×0.6 m.); *P*, wooden platform (2×1 m.); *R*, conical tinned reflector; *S*, cloth screen; *B*, bracket for holding screens; *O*, observer.

black paper and taken into the laboratory. Here the fireflies were left in darkness for various lengths of time and then put into the cage (twenty-five at a time, i.e., the contents of one bottle) and exposed to various intensities of light and to various changes in illumination. They were closely observed under all of these conditions, and the total number of flashes per minute per twenty-five individuals ("frequency of flashing"[3]) was recorded. The results obtained are presented below.

THE EFFECT OF BRIGHT LIGHT ON FLASHING

In the laboratory different groups of fireflies were exposed to 2,500±m.c. for periods ranging up to 12 hours, to 800±m.c. for periods ranging up to 18 hours, and to 320±m.c.

[3] In most of the figures, for the sake of simplicity, the frequency of flashing is plotted as the average numbers of flashes per minute per twenty-five individuals in successive 5-minute periods, instead of as the actual numbers per minute.

for considerable periods of time. No flashing was observed in these intensities. In addition, fireflies which were flashing in dim light or in darkness were exposed to bright light, and it was found that all flashing ceased immediately. It is consequently concluded that bright light prevents flashing. The lower limit of intensity which prevents flashing was not precisely ascertained, but it is between 320 and 210 m.c. under ordinary conditions.[4]

THE EFFECT OF DARKNESS ON FLASHING

Effect of darkness following bright light.—Fireflies were subjected to darkness for various periods, then exposed in the cage to bright light (2,500±m.c.) for 15 minutes, and then subjected to darkness again (i.e., previous time in darkness varied, time in bright light constant). Figure 3 presents the results of several experiments of this type. This

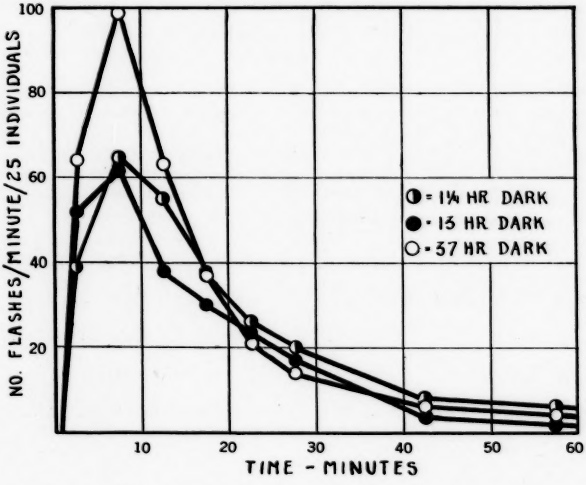

FIG. 3.—Relation between flash frequency and time in darkness preceding exposure to bright light. Fireflies were subjected to darkness for 1¼ hours (five experiments), 13 hours (two experiments), and 37 hours (two experiments), then to bright light for 15 minutes, then to darkness again, in which the frequencies of flashing were measured. Ordinates, average numbers of flashes per minute per twenty-five individuals.

figure shows that, when fireflies were exposed to bright light for 15 minutes and then subjected to darkness, the frequency of flashing increased precipitously to a maximum in about 8 minutes, then decreased rapidly for about 15 minutes, and thereafter decreased more gradually nearly to zero at the end of an hour. It shows also that the frequency of flashing in darkness following 15 minutes' exposure to bright light was greater if the fireflies had been previously subjected to darkness for 37 hours than it was if they had been subjected to darkness for 13 hours or for 1¼ hours.

[4] The fact that the fireflies ordinarily do not flash in the field during the day (intensities up to 100,000 m.c.) shows that there can be enormous variation in light intensity without flashing. Harvey (1926) reported that flashing in *Photuris pennsylvanica* was inhibited by exposure to 15,000 foot candles (160,000 m.c.) if the luminous organ alone was illuminated, but not if the eyes were illuminated while keeping the luminous organ in the dark.

In another series of experiments groups of fireflies were subjected to darkness for a given time; then some of them were exposed to bright light for 15 minutes, some for 30 minutes, others for 1 hour, others for 2 hours, and still others for 4 hours, after which they were subjected to darkness again (i.e., previous time in darkness constant, time in bright light varied). In these experiments it was found that flashing occurred whenever the intensity was changed from bright light to darkness, but that no consistent difference in the frequency of flashing in darkness was observed following the different times of exposure to bright light.

Effect of darkness following dim light.—Fireflies were exposed to dim light ($65 \pm$ m.c.) and left until flashing became infrequent or ceased, and were then subjected to darkness.

TABLE II

EFFECT ON FREQUENCY OF FLASHING OF REDUCTION IN INTENSITY FROM DIM LIGHT TO DARKNESS

Note that increase in frequency resulted.

Experiment	Number of Flashes per Minute in Dim Light in 5 Consecutive Minutes Previous to Subjection to Darkness	Average Flashes per Minute in Darkness	Number of Flashes per Minute in Darkness Following Reduction in Intensity from Dim Light to Darkness	Average Flashes per Minute in Dim Light
1	8, 2, 3, 0, 4, ...	3	4, 3, 7, 19, 16, 14, ...	10
2	4, 10, 3, 2, 3, ...	4	15, 17, 23, 21, 24, ...	20
3	1, 2, 2, 0, 0, ...	1	18, 22, 30, 20, ...	22
4	3, 3, 4, 0, 5, ...	3	8, 17, 10, 27, 21, ...	17
5	0, 0, 0, 0, 0, ...	0	4, 21, 21, 21, 16, 24, 21, ...	18
6	0, 0, 0, 0, 0, ...	0	0, 2, 11, 13, 11, 14, ...	8
7	0, 0, 0, 0, 0, ...	0	13, 29, 34, 32, 36, 35, 33, 25, 30, 38, 23, 21, 19, 17, 18, 15, 11, 7, 10, 5, 5, 10, 4, ...	20

The frequency of flashing was measured for 5 consecutive minutes previous to the exposure to darkness, and also for various periods after the change. Table II contains the results of seven such experiments. This table shows that reduction in intensity from dim light to darkness at once produced a very marked increase in the frequency of flashing, and that the flashing continued only a short time (Table II, Exp. 7).

Additional experiments showed that the lower the intensity of the dim light, the smaller the increase in the frequency of flashing resulting from reduction in intensity from it to darkness, and that exposure to darkness does not cause flashing in fireflies which have been in dim light for a considerable time without flashing.

Effect of continuous darkness.—Fireflies were brought in from the field in the evening in bottles, as described. Some of the bottles were unwrapped (not opened) in the darkroom in total darkness (three experiments); other bottles were emptied into the cage in total darkness (four experiments); one other was emptied into a large, covered glass

aquarium. In each of these eight experiments the number of flashes produced by the 25 fireflies was recorded for 5 consecutive minutes every half-hour or hour for 28 consecutive hours. The average of the results of these experiments is presented in Figure 4. This figure shows that the frequency rose to a maximum of about 5 flashes per minute per twenty-five individuals at the end of a half-hour; then, in another hour, declined practically to zero. Figure 4 shows also that the frequency remained at this low level (only an occasional flash) for about 20 hours, after which it rose again to about 2 flashes per minute and, after 3 or 4 hours, decreased again to zero. The significance of the slight increase in frequency which occurred when the fireflies had been in darkness about 23 hours will be considered later.

Summary.—The results presented show that change from bright light to darkness induces flashing; that change from dim light to darkness temporarily increases the frequency of flashing; and that the frequency of flashing in continuous total darkness is relatively very low.

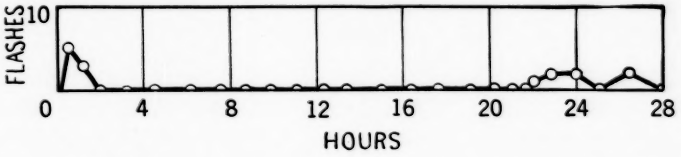

FIG. 4.—Frequency of flashing in continuous total darkness. Average of results of eight experiments. Ordinates, average numbers of flashes per minute per twenty-five individuals. Note that the frequency of flashing is relatively very low.

THE EFFECT OF DIM LIGHT ON FLASHING

Effect of dim light following bright light.—Fireflies were exposed to bright light (800± and 2,500±m.c.) for periods ranging from 15 minutes to 12 hours; then the intensity was reduced to that of dim light. In every test they immediately became active and flew about and flashed in an entirely normal manner regardless of the time of day. The fact that a sufficient reduction in intensity induces flashing at any hour of the day appears to support the contention of Rau that, in the field, flashing is correlated with definite light intensities; and it appears to offer no support to the view of Mast that the flashing is a periodic phenomenon.

Effect of dim light following darkness.—Bottles containing fireflies were taken into the laboratory, as described, and left in darkness for some time. Then the twenty-five fireflies from one of them were put into the cage and exposed to dim light (65±m.c.), and the frequency of flashing was recorded for an hour or more. Finally the fireflies in the cage were replaced by twenty-five from another bottle which had been in darkness longer, and the frequency was again measured. This general procedure was repeated with each group of fireflies.

Figure 5 presents the detailed results of three typical experiments. This figure shows that fireflies which were left in darkness for 1, 4, or 12 hours and then exposed to dim light began to flash almost at once, and that the frequency of flashing increased most rapidly and rose highest after 1 hour, and least after 12 hours, in darkness.

Figure 6 shows the results of forty-three experiments (similar to the three described above) in each of which the frequency of flashing in dim light was measured following

one of fifteen different periods in darkness. In this figure each point represents the average number of flashes per minute per twenty-five individuals for all the experiments performed with a particular period of subjection to darkness. Figure 6 shows the following: The frequency of flashing in dim light following, respectively, 1, 2, and 4 hours in darkness did not differ greatly, averaging about 45 flashes per minute; but thereafter it decreased markedly to about 11 flashes per minute following 8 hours in darkness, 3 flashes per minute following 12 hours in darkness, and practically to zero following 18 hours. After 24 hours in darkness the frequency was extraordinarily high, compared with the frequencies following 8, 12, and 18 hours. After 36 hours in darkness the frequency was low; after 48 hours it was high; after 60 hours it was low; after 72 hours, high; after 84 hours, low; after 96 hours, high. Each period of high frequency occurred approximately at 8:00 P.M.

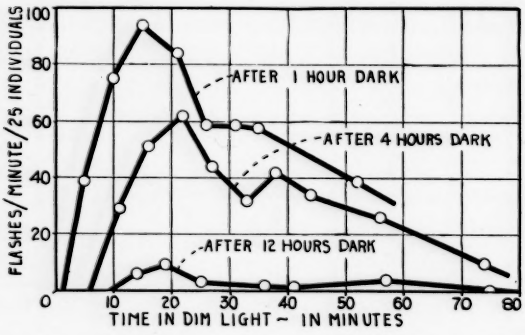

FIG. 5.—Frequency of flashing in dim light following various periods in darkness. Ordinates, average numbers of flashes per minute per 25 individuals.

These results show that, as the time in darkness previous to subjection to dim light increases, the frequency of flashing follows a definite cycle, in which periods of great flashing activity occur at 24-hour intervals, and that these periods alternate with periods of inactivity. Since the conditions in the darkroom under which the fireflies flash (following, e.g., 24 and 48 hours in darkness) are apparently precisely the same as those under which they do not flash (following, e.g., 36 and 60 hours in darkness), these results indicate that there actually is, as Mast maintained, an inherent diurnal periodicity in the flashing of these fireflies and that this periodicity persists for at least 4 days under the constant conditions of the darkroom. However, the fact that in the experiments described the periods of high frequency all occurred at about 8:00 P.M., as they do in the field, suggested that the periodicity might have been correlated with some unknown daily periodic change in the environment (e.g., cosmic rays) rather than with inherent periodic changes within the firefly. The results obtained in the following experiments concern this possibility.

Fireflies brought from the field at 8:00 P.M., as usual, were exposed immediately to bright light (2,500±m.c.) and left 12 hours (i.e., until 8:00 A.M.); then some were subjected to darkness for 12 hours (three experiments), and others for 24 hours (two experiments), after which they were exposed to dim light. Those which had been in darkness for 12 hours did not flash when exposed to dim light, although it was then 8:00 P.M.; but those which had been in darkness for 24 hours flashed vigorously, although it was then 8:00 A.M. The whole cycle had thus been reversed with respect to the time of day. In another similar series of experiments fireflies were exposed to 18 hours of bright light followed by 12 and 24 hours of darkness and then dim light. Again, those which had been in darkness 12 hours (i.e., until 2:00 A.M.) did not flash when exposed to dim light; whereas those which had been in darkness 24 hours flashed at once.

The results of these experiments show that the 24-hour cycle of activity can begin at any time of day, and consequently that it is not correlated with any periodic environmental change. It must, therefore, be correlated with a rhythm, inherent in the firefly, which under appropriate conditions persists regardless of the time of day. Confirmatory evidence concerning this conclusion is presented in the following paragraphs.

Effect of continuous dim light.—In the experiments thus far described concerning periodicity (e.g., Fig. 6), the flashing occurred in response to a *change* in light intensity from darkness to dim light. The question now arises as to what occurs in continuous illumination, or, in other words, whether or not the fireflies ever begin to flash of their own accord without any change in illumination.

Several groups of fireflies were brought from the field at 8:00 P.M., subjected to darkness, and left there, some for 1 hour, some for 12 hours, others for 36, others for 60, and

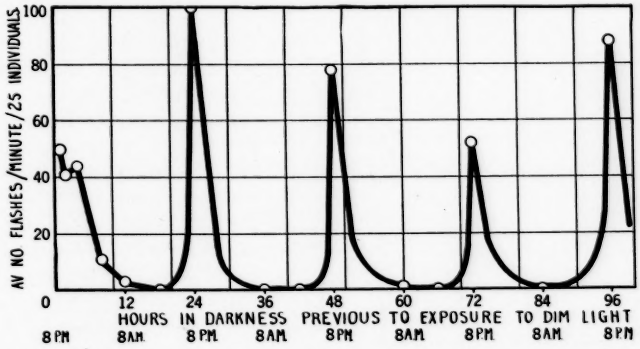

FIG. 6.—Frequency of flashing in dim light following various periods in darkness. Ordinates, average numbers of flashes per minute per twenty-five individuals in first 30 minutes of each experiment. Each point represents the average of several experiments, as follows: 1 hour, 2 experiments; 2 hours, 4; 4 hours, 5; 8 hours, 1; 12 hours, 5; 18 hours, 1; 24 hours, 2; 36 hours, 8; 48 hours, 1; 60 hours, 5; 72 hours, 1; 84 hours, 3; 96 hours, 1. Note the cycles of activity in which periods of high flash frequency recur at 24-hour intervals.

still others for 84 hours. Then they were exposed to dim light and left for 12 or more hours continuously, during which time the frequency of flashing was measured and recorded at regular intervals. Figure 7 illustrates the result of an experiment in which fireflies were exposed to dim light following 1 hour in darkness, and the frequency of flashing measured for more than 26 hours. This figure shows that the twenty-five fireflies exposed to dim light at about 9:00 P.M. flashed for 2 or 3 hours at a frequency of about 20 flashes per minute; then stopped and did not flash again throughout the rest of the night and the entire next day; but that at about 6:00 P.M. of the second evening they suddenly began to flash again spontaneously (i.e., without any change in light intensity), and that the frequency rose abruptly to over 120 flashes per minute and then decreased relatively rapidly nearly to zero.

Figure 8 shows the results of typical experiments in which the frequencies of flashing in dim light were measured following, respectively, 1, 12, 36, 60, and 84 hours in darkness. These curves show that following 12, 36, 60, and 84 hours in darkness the frequency of flashing in dim light remained practically at zero for about 12 hours and then

suddenly, without any change in the environment, increased enormously at approximately 24, 48, 72, and 96 hours, respectively, after the beginning of the experiments.

The results presented in Figures 7 and 8 confirm the conclusion previously reached that there is an inherent diurnal periodicity in the flashing, and show, in addition, that under appropriate conditions the periodicity is manifested by periods of flashing activity which occur at 24-hour intervals without any environmental change.

In a relatively small proportion of the experiments the expected recrudescence of flashing occurred prematurely, i.e., the flashing began earlier than would be expected if there is a 24-hour cycle. However, this did not occur in any of the experiments in which the fireflies were transferred to the cage from the bottles in total darkness several hours previous to the time at which they were exposed to dim light. The anomalous results were therefore probably due to mechanical stimulation of the fireflies caused by the transfer to the cage at the time they were exposed to dim light.

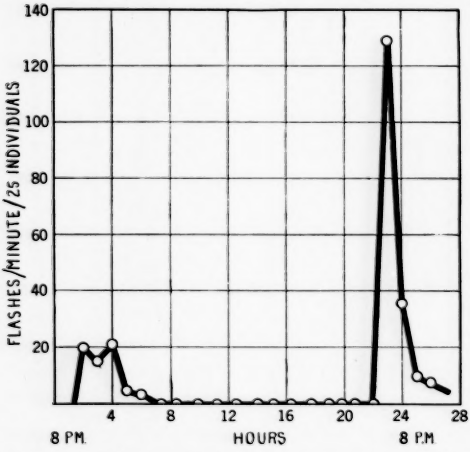

FIG. 7.—Frequency of flashing in continuous dim light following 1 hour in darkness. Frequency measured continuously for 26 hours. Note that a spontaneous recrudescence of flashing occurred after the fireflies had been in dim light for about 22 hours.

By referring to Figure 4, it will be seen that in continuous darkness there was a slight increase in flash frequency about 22 hours after the beginning of the experiments. The increase is too slight to be very significant, but the fact that it occurred in several experiments suggests that the 24-hour cycle persists to some extent even in total darkness.

THE EFFECTS OF SMALL CHANGES IN LIGHT INTENSITY ON FLASHING

Fireflies in the cage were exposed for some time to one of various intensities of dim light (3.2–130 m.c.); the frequency of flashing was recorded; and the intensity was then reduced or increased slightly and the frequency again recorded. The results obtained are presented in Table III, which shows that, between the limits of light intensity within which flashing occurs, reduction in intensity (Expts. 1, 5, 7) caused increase in frequency of flashing, whereas increase in intensity (Expts. 2, 3, 4, 6) caused decrease in flashing. This table shows also that there was no strict proportionality between the magnitude of the change in light intensity and the magnitude of the resulting change in frequency of flashing, although the general effects were unmistakable and in harmony with the conclusions already reached.[5]

MISCELLANEOUS INFLUENCES ON FLASHING

Temperature.—It has already been shown that temperature has an effect on the time of flashing. Snyder and Snyder (1920) found that increase in temperature resulted in

[5] It is interesting to note that whereas the intensity of full moolight is only about 0.24 m.c., the amount of flashing is very conspicuously less on moonlit nights than on moonless ones.

an increase in the frequency of flashing, and that flashing occurred only above 17° C. The writer confirmed these results.[6] However, since the same temperature may prevail at times when no flashing is occurring, as well as when flashing is occurring, it is obvious that flashing is not specifically correlated with temperature.

Humidity.—It was found that low humidity inhibits flashing through its injurious effect on the general vitality of the fireflies. For this reason the darkroom was artificially humidified during experiments involving more than a few hours' observations.

Experiments were performed to ascertain the effect of high humidity on flashing. Fireflies were placed in bottles in some of which the humidity was favorable and in

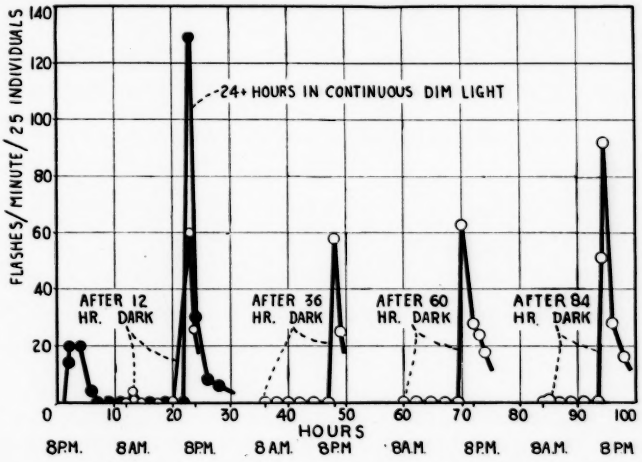

FIG. 8.—Frequency of flashing in continuous dim light following various periods in darkness. ●, exposed to dim light after 1 hour in darkness; ○, exposed to dim light after 12, 36, 60, and 84 hours in darkness. Number of experiments performed for each period of darkness: 1 hour, 1; 12 hours, 2; 36 hours, 7; 60 hours, 5; 84 hours, 1. Note the spontaneous bursts of flashing at about 24, 48, 72, and 96 hours after the beginning of the experiments.

others of which the air was saturated with water vapor (25°). The bottles were exposed to bright light for 75 minutes; then the fireflies were transferred to the cage, subjected to darkness, and the frequency of flashing was measured. The results obtained are presented in Figure 9, which shows that the fireflies which had been in very humid air flashed at a much lower frequency than those which had been in drier air. This depression in frequency was probably due to condensation of water vapor on the wings, which prevented flying and, secondarily, flashing.

Both low and high humidity thus decrease the frequency of flashing.

Air currents.—In the field it was often observed that wind affected the flashing of males in flight by blowing them off their course, causing a prolongation of the intervals between successive flashes (decreasing the frequency). In the laboratory, fireflies were exposed to dim light for some time, and their frequencies of flashing were measured. Then a gentle current of air (not strong enough to visibly interfere with flight) was caused

[6] Forthcoming publication.

TABLE III

EFFECT OF SMALL CHANGES IN LIGHT INTENSITY ON FREQUENCY OF FLASHING

The frequency of flashing is given as the number of flashes per minute per twenty-five individuals in consecutive minutes. Note that increase in intensity caused decrease in frequency of flashing, and vice versa.

EXPERIMENT	CHANGE IN LIGHT INTENSITY (METER-CANDLES)		FREQUENCY OF FLASHING BEFORE CHANGE	AVERAGE FREQUENCY BEFORE CHANGE	FREQUENCY OF FLASHING AFTER CHANGE	AVERAGE FREQUENCY AFTER CHANGE
	From	To				
1.....	27	10	5, 11, 16, 10, 3, ...	15	24, 22, 25, 24, 19, ...	23
2.....	10	27	8, 5, 9, 12, 6, ...	8	3, 4, 7, 2, 1, 0, 0, 1, 1, 4, 0, ...	2
3.....	65	130	4, 15, 5, 8, 10, ...	8	0, 0, 0, 0, 1, ...	0.2
4.....	27	54	18, 17, 18, 12, 18, ...	16	1, 4, 5, 4, 6, ...	4
5.....	54	27	20, 21, 16, ...	19	24, 22, 34, 37, 30, ...	29
6.....	3	14	2, 0, 2, 1, 5, ...	2	0, 0, 0, 0, 1, 0, ...	0.2
7.....	14	3	0, 0, 3, ...	1	12, 21, 18, 23, 27, ...	20

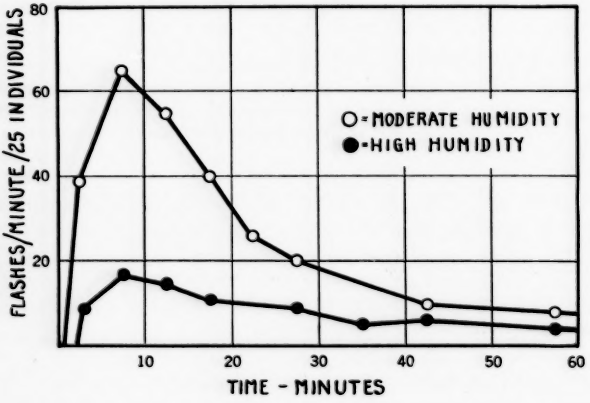

FIG. 9.—Effect of humidity on frequency of flashing. Upper curve, average frequency for five experiments in moderate humidity; lower curve, frequency in similar experiment in high humidity. Note that the high humidity decreased the frequency of flashing.

to circulate through the cage by means of an electric fan, and the frequency was again measured. The results obtained in three such experiments are presented in Table IV. This table shows that the current of air caused a considerable increase in the frequency of flashing.

TABLE IV

EFFECT OF AIR CURRENTS ON FLASHING

Frequency of flashing given as the number of flashes per minute per twenty-five individuals. Average lapse of 14 minutes between the time the ventilator was turned on and the time the frequency was measured again. Note that the currents of air resulted in increased frequency.

Experiment	Frequency of Flashing in Calm	Average Frequency in Calm	Frequency of Flashing Following Air Current	Average Frequency after Air Current
1.....	26, 22, 31, 30, 21, ...	26	57, 43, 46, 49, 47, 63, 48, ...	50
2.....	19, 18, 17, 12, 9, ...	15	37, 26, 36, 38, 19, 19, ...	29
3.....	0, 0, 1, 0, 0, 1, ...	0.3	8, 4, 0, 2, 4, ...	3

SUMMARY

1. Observations were made in the field by measuring various environmental factors and ascertaining their effects on flashing. Experiments were performed in the laboratory by subjecting fireflies to various changes in light and other agents and ascertaining their effects on flashing under controlled conditions.

2. In the field the time at which males of the firefly *P. pyralis* begin to flash is correlated with temperature and light intensity but not specifically with the time of day.

3. Bright light prevents flashing.

4. Change from bright light to darkness induces flashing; change from dim light to darkness causes a temporary increase in the frequency of flashing; the frequency of flashing in continuous total darkness is relatively very low.

5. Change from bright light to dim light induces flashing at any hour of the day, indicating that the response is correlated with a specific intensity of light.

6. Change from darkness to dim light induces flashing provided the fireflies have previously been in darkness for 24, 48, 72, or 96 hours, but not if they have been in darkness 12, 36, 60, or 84 hours.

7. Fireflies exposed to continuous dim light begin to flash spontaneously (i.e., without any environmental change) when the sum of the time spent in dim light and the time previously spent in darkness is equal approximately to 24 hours.

8. The periods of flashing activity which recur at 24-hour intervals may be made to occur at any hour of the day, showing that the periodicity in flashing is not correlated with any periodic change in the environment.

9. There exists in *P. pyralis* an inherent diurnal periodicity which is manifested by periods of flashing which recur at 24-hour intervals, and which persists for at least 4 days in the uniform conditions of the darkroom.

10. Between the limits of light intensity within which flashing occurs, reduction in

intensity results in increase in frequency of flashing, and increase in light intensity results in decrease in frequency of flashing.

11. The frequency of flashing is increased by increase in temperature and by gentle air currents, and decreased by high or low humidity.

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EFFECTS OF AGE ON ARBACIA EGGS. VI. INCREASED STRETCHING AND BURSTING OF EGG MEMBRANE IN HYPOTONIC SEA WATER

(Five figures)

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IN PREVIOUS studies unfertilized eggs of *Arbacia punctulata*, aged in sea water, progressively increased in size (Goldforb, 1935*a*) and in viscosity (Goldforb, 1935*c*). Permeability to water, calculated by the graphic method of Lucké and McCutcheon (1926) and McCutcheon and Lucké (1926), or, by the integration method of Northrop (1927), also increased progressively with age (Goldforb, 1935*b*). This increasing permeability was confirmed, and other effects of aging were disclosed, by a study of the bursting rate in hypotonic sea water.

PROCEDURE

The experiments were performed at the Marine Biological Laboratory at Woods Hole, Massachusetts. Dr. V. Schechter assisted in the bursting experiments in 1930; M. Landowne, in 1931 and 1932. *Arbacia* were freshly collected, and the eggs of each female tested separately. If in good condition, the eggs were washed, transferred to flat bowls, and the sea water changed twice daily. The temperature ranged from 19° to 22.5° C., but within 2° during each experiment.

At each age, 0.1 cc. egg suspension was transferred to 15 cc. hypotonic sea water of the same concentration, pH, and temperature $\pm 0.5°$ C. The time for 50 per cent to burst was the criterion of change. The physiologic condition at each age was determined by the percentage and normality of cleavage, after insemination with freshly prepared 1 per cent suspension of fresh dry sperm.

WIDE VARIATION IN BURSTING TIME OF EGGS FROM DIFFERENT FEMALES

Freshly shed eggs from different females, subjected to the same conditions, burst at widely varied rates. Table I is illustrative. In Experiment 1 the eggs from 7 females were tested in 20 per cent sea water at 19° C. The time for 50 per cent to burst varied from 5 min. 40 sec. to 11 min. 20 sec., or 100 per cent. To minimize the effect of age, the eggs of 4 females were tested within 13 min. (Expt. 2). Bursting time ranged from 1 min. 56 sec. to 3 min. 48 sec., or 96 per cent. Similar wide variations occurred in repeated experiments.

This wide variation was not due to differences in age or physiologic condition; nor was it due to experimental error. Repetitive tests varied by 0 to 8, average 3.0 per cent. Wide variations also occurred in thickness of jelly layer and size of eggs (Goldforb, 1935*a*), in viscosity (Goldforb, 1935*c*), permeability to water (Goldforb, 1935*b*), physiologic condition (Goldforb, 1917, 1918), and agglutination of sperm (Goldforb, 1929).

DECREASED BURSTING TIME WITH AGE

Eggs from the same female, tested by the same procedure, burst progressively faster at successive ages. Figure 1 is illustrative. The eggs, freshly shed and in excellent condition, were tested in 30 per cent sea water at 21.5° C. The first egg burst, at successive ages, in decreasing time, namely, from 8 min. 55 sec. to 3 min. 10 sec. The time for 10 per cent to burst decreased from 10 min. 20 sec. to 3 min. 45 sec. The time for 50 per cent to burst decreased from 15 min. 55 sec. to 4 min. 30 sec. Ninety per cent did not burst during the 25-min. observation at the first two ages. Subsequently bursting decreased from 9 min. 00 sec. to 5 min. 15 sec. And for these latter ages the time for all

TABLE I

VARIATION IN BURSTING TIME OF EGGS FROM DIFFERENT FEMALES
(0.2 cc. Eggs in 15 cc. of 20 Per Cent Sea Water at 19° C. [Expt. 1] and 22° C. [Expt. 2])

Expt. No.	♀ No.	Age	Time for 50 Per Cent To Cytolize	Percentage of Cleavage
1	3	47 min.	5 min. 40 sec.	90
	4	52	6 25	98
	2	30	7 15	95
	6	35	8 30	*
	5	20	9 30	*
	1	19	10 0	96
	7	51	11 20	*
	Variation 100 per cent			
2	2	5½ hr.	1 min. 56 sec.	92
	4	5½	2 5	89
	1	5½	2 50	93
	3	5½	3 48	100
	Variation 96 per cent			

* In distilled water 95 or more per cent formed parthenogenetic membranes.

eggs to burst decreased from 10 min. 00 sec. to 5 min. 52 sec. When 28 hr. old the eggs burst about three times faster than when 4 hr. old. In other experiments bursting increased much more with age.

BURSTING VERSUS DETERIORATION

Bursting time, as well as physiologic condition of the eggs, changed with age. But the two curves were quite different. This is illustrated by Figure 2. Eggs from one female were tested in 30 per cent sea water between 2½ and 71 hr. after shedding. Bursting time (for 50 per cent of the eggs) decreased progressively with age, from 12 min. 12 sec. to 2 min. 21 sec. Cleavage likewise decreased from 98 to 10 per cent. It will be noted however that, at the earliest ages, when the eggs were in best condition and deteriorating least rapidly, the bursting time changed maximally, with age. During intermediate

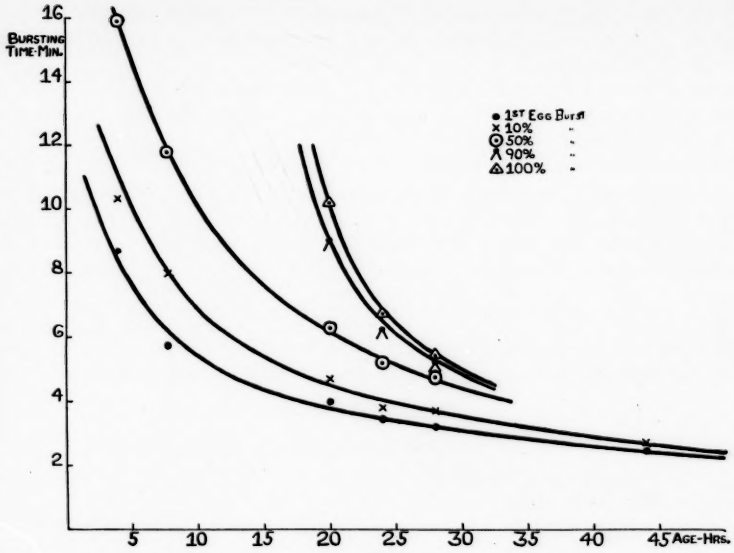

FIG. 1.—Eggs burst more rapidly with age. The five curves represent bursting time of the first egg, and of 10, 50, 90, and 100 per cent of the eggs, at successive ages. Eggs from one female, tested in 30 per cent sea water, 21.5° C., pH 8.3.

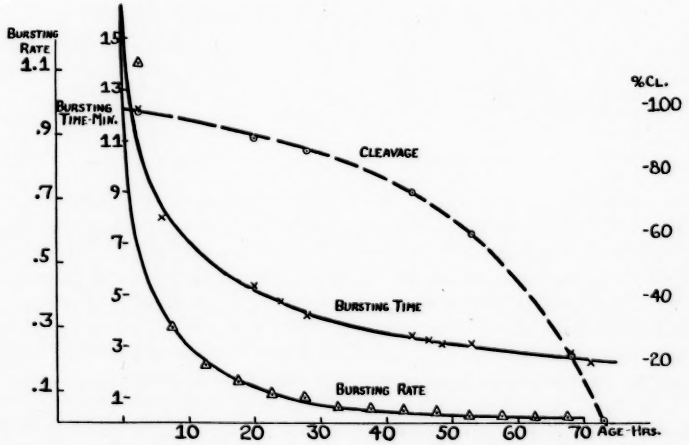

FIG. 2.—Bursting time, of 50 per cent of each sample, decreased sharply with age. Bursting rate also shown. Bursting changed maximally during least deterioration, and vice versa. Eggs, from one female, tested in 30 per cent sea water, 21.5° C., pH 8.3.

ages, when eggs were deteriorating more and more rapidly, the bursting time changed less rapidly. During late ages, when deterioration was greatest and most rapid, bursting time decreased minimally.

Bursting rate is also plotted in Figure 2, i.e., at successive 5-hr. intervals. During earliest ages this rate changed even more rapidly than did the bursting time. During late ages the bursting rate was almost constant.

Deterioration and bursting were inversely proportional. Bursting time changed most rapidly when eggs were least deteriorated, and vice versa. These results, in repeated experiments, suggested that bursting time or rate was a function not of deterioration per se but of other factors. The following experiments demonstrate that increased stretching of the plasma membrane is one of the crucial factors.

INCREASED STRETCHING OF MEMBRANE WITH AGE

When measured for 5 consecutive minutes in 60 per cent sea water at constant temperature, the eggs swelled and their membranes were stretched increasingly with age.

TABLE II

EXPERIMENT E: SWELLING OF EGGS (V_5–V_0) AND STRETCHING OF MEMBRANE (S_5–S_0) WITH AGE

(Eggs from 1 Female in 60 Per Cent Sea Water at 21.5° C. for 5 Min.)

Age (Hr.)	No. of Eggs	Swelling in 5 Min. (V_5–V_0) (μ^3)	Stretching in 5 Min. (S_5–S_0) (μ^2)	Percentage of Cleavage
1	16	67,900	3,520	100
5	15	70,100	3,640	99
9	19	70,000	3,600	97
24	21	74,200	3,800	86
32	18	74,900	3,830	74
47	14	76,200	3,905	27
Increase		8,300	385	
Percentage of increase		12.2	10.9	

Methods of measurement, technique, and experimental error were the same as in a previous study (Goldforb, 1935*b*). In Table II, swelling, during 5 min., increased with age from 67,900μ^3 to 76,200μ^3, or 12.2 per cent. The plasma membranes were stretched increasingly, from 3,520μ^2 to 3,905μ^2, or 10.9 per cent. In similar experiments there was even greater stretching (Table III). In Experiment D stretching increased with age from 3,880μ^2 to 5,180μ^2, or 33.5 per cent. In other experiments it increased 21.9, 29.7, and 39.6 per cent, respectively.

Stretching to equilibrium.—Membranes were measured at each age in 60 per cent sea water until the eggs were definitely in equilibrium with the solution. Higher concentrations were not used because of decreased accuracy in measuring. Concentrations below 50 per cent induced injury (Lucké and McCutcheon, 1930). Measurements were made each minute for 5 min.; then 2–4 min. apart thereafter. In Table IV, when the eggs ceased swelling, their membranes averaged 19,800μ^2. This equilibrium size was prac-

tically the same at successive ages. The amount of membrane stretching, from initial to equilibrium size, was also the same at all ages, viz., *ca.* 3,600μ^2. But equilibrium was reached more quickly in older eggs. When 2 hr. old, equilibrium was not quite reached

TABLE III

STRETCHING OF PLASMA MEMBRANE WITH AGE

(Conditions Same as in Table II)

Female	No. of Eggs Measured	Percentage of Sea Water Used	Measured at Following Ages (Hr.)	Percentage of Increase in Amount and Rate of Stretching
H	65	60	1, 6, 22, 30, 48*	39.6
D	100	50	1, 3, 6, 24, 28, 32, 48*	33.5
I†	48	60	3, 7½, 21, 27, 44*	29.7
K	59	80	1½, 5, 10, 23, 30*	21.9

* Age when maximum stretching was observed.
† Measured for 7 min. at each age.

TABLE IV

EXPERIMENT I: AMOUNT OF STRETCHING, RATE OF STRETCHING, EQUILIBRIUM PERIOD, AND BURSTING RATE

(Eggs from 1 Female in 60 per Cent Sea Water at 21.5° C. pH 8.3)

Age (Hr.)	No. of Eggs Measured	Size of Membrane after 30 Min. in Solution (μ^2)	Amount Stretched ($S_{30}-S_0$) (μ^2)	Minutes To Reach Equilibrium	Stretching Rate		Bursting Rate		Duration of Equilibrium (Min.)	10 per Cent Burst
					μ^2/Min.	Percentage of Increase	Minutes for 10 Percent To Burst	Percentage of Increase		
2	12	19,800	3,600	>30	120	.....	135	.....	25	75th min. of secondary stretching
7½	14	19,800	3,610	25	144	20	.....	.....	.....	
21	14	19,770	3,570	16	223	95	52	260	16	20th min. of secondary stretching
27	10	19,660	3,560	13	274	128	31	430	11	7th min. of secondary stretching
44	5	20,370* 19,600†	4,370* 3,600†	11½	380* 313†	216* 160†	10	1,350	0	1½ min. before equilibrium

* Increase due to secondary stretching.
† Based upon previous equilibrium size.

after 30 min. in the solution. At successive ages, equilibrium was reached after 25, 16, 13, and 11½ min., respectively. Hence, the stretching rate increased sharply with age, i.e., from 120μ^2 to 313μ^2 per minute, or 160 per cent.

In a similar experiment the amount of stretching to equilibrium was 3,850μ^2. This amount did not change with age (i.e., only 0.7 per cent). Rate of stretching, however, whether measured as in the previous experiment or, more accurately, as in Table V, markedly increased with age. At successive ages the time required to stretch the same submaximal amount (viz., 3,000μ^2) decreased with age, from 12.0 to 5.3 min., respectively (Table V). The stretching rate increased from 250μ^2 to 566μ^2 per minute, or 126 per cent.

TABLE V

EXPERIMENT Q: TIME REQUIRED TO STRETCH 3,000 μ^2 AT SUCCESSIVE AGES

(Eggs from 1 Female in 60 per Cent Sea Water at 21° C., measured for 60–105 Min.)

Age (Hr.)	Time To Stretch 3,000 μ^2 (Min.)	Stretching Rate (μ^2/Min.)
1½	12.0	250
13½	8.0	375
25	7.5	400
35	5.3	566
Percentage of increase		126

When 50 per cent sea water was used, the amount of stretching to equilibrium was 8,300μ^2. This amount did not alter significantly with age. But the rate of stretching increased from 457μ^2 to 1,042μ^2 per minute, or 128 per cent (Table VI).

TABLE VI

EXPERIMENT R: TIME REQUIRED TO STRETCH 7,000 μ^2 AT SUCCESSIVE AGES

(Eggs from 1 Female in 50 per Cent Sea Water at 21° C.)

Age (Hr.)	Minutes To Stretch 7,000 μ^2	Average Stretching Rate (μ^2/Min.)
⅙	15.3	457
6	11.8	593
12	12.3	569
21	9.7	722
31	6.7	1,042
Percentage of increase		128

Equilibrium and subsequent stretching.—The lower curves of Figure 3 represent membrane stretching of 3 eggs 1½ hr. old tested in 60 per cent sea water for 95 min. These curves are typical. They show (1) an initial or primary stretching during the first 25 min. (approx.), (2) an equilibrium phase during the next 25 min. (approx.), and (3) a final phase, or secondary stretching, during the last 45 min. The upper curves of Figure 3

represent the stretching curves of 3 eggs in 50 per cent sea water. The same three phases occur, though less sharply defined. Leitch (1935) reported similar phases in *Echinometra* eggs.

In the foregoing experiments neither primary nor total amount of stretching (for 95 min.) increased with age. Stretching rate, however, increased in the first and last phases. Rate of primary stretching increased 126 and 160 per cent, respectively, with age. Rate of secondary stretching increased 100 and 29 per cent, respectively.

Not only was equilibrium reached more quickly with age, but the duration of equilibrium was progressively reduced to zero. At late ages, therefore, secondary stretching followed directly upon primary stretching, without the intermediate equilibrium phase.

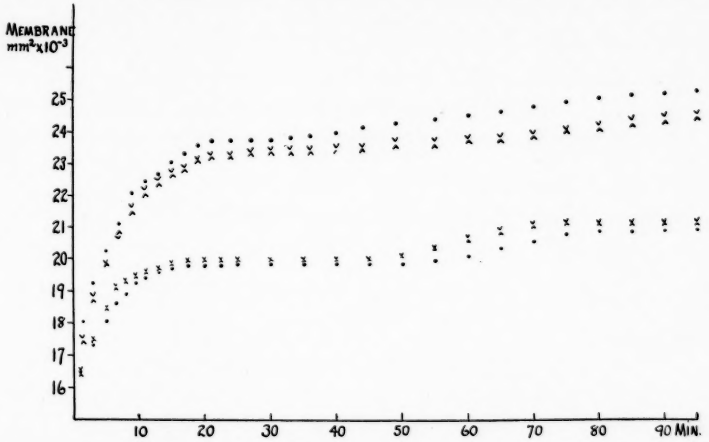

FIG. 3.—Lower set of three curves represents characteristic membrane stretching of 3 eggs, 1½ hr. old, in 60 per cent sea water, 21° C. Each curve shows a primary stretching, equilibrium, and secondary stretching. The upper set of three curves shows similar tripartite curves, of 3 eggs, ½ hr. old, in 50 per cent sea water, 21° C.

INCREASED STRETCHING AND BURSTING RATES COMPARED

Since stretching and bursting rates increased with age, it was desirable to determine these rates for eggs of the same female, tested at the same ages and under the same conditions. In Table IV the stretching rate, between 2 and 21 hr., increased from 120μ^2 to 223μ^2 per minute, or 95 per cent. The bursting rate increased much more, viz., 260 per cent. Between 2 and 27 hr., stretching rate increased 128 per cent; bursting rate, 430 per cent. Between 2 and 44 hr., stretching rate increased 160 per cent; bursting rate, 1,350 per cent. Each increase in stretching rate, with age, resulted in 2.7–8.4 times greater bursting rate. The greater the stretching rate, the disproportionately greater was the bursting rate. Similar results were obtained in repeated experiments.

It was previously shown that permeability to water increased at progressively faster rates with age (Goldforb, 1935*b*). It is this increasing rate of water intake which is held responsible for the faster swelling and membrane stretching and the consequent disproportionately increased bursting rate of aging eggs.

STRETCHING AND BURSTING AT INCREASING OSMOTIC PRESSURES

The phenomena described above also occurred when the rate of water intake was increased, not by age, but by greater osmotic pressure.

At a given age an increase in osmotic pressure resulted in a slight increase in amount of stretching but a marked increase in rate of stretching. For example, in Table VII, eggs of the same age were tested in 50 and in 40 per cent sea water. The amount of primary stretching increased only 3.9 per cent. Total amount of stretching, for 70 min., increased 10.3 per cent. Rate of primary stretching, however, increased 164 per cent.

Increasing the osmotic pressure at a given age disproportionately accelerated the bursting rate. The resulting curve (Fig. 4) is essentially the same as the bursting curve, at a constant osmotic pressure and at successive ages (see Fig. 1 and 2). In Figure 4 the

TABLE VII

EXPERIMENT R: EFFECT OF INCREASED OSMOTIC PRESSURE ON AMOUNT AND RATE OF MEMBRANE STRETCHING

(Eggs 22 hr. old, from 1 Female, Measured in 50 and 40 per Cent Sea Water at 21° C.)

Percentage of Sea Water	Membrane at Equilibrium (μ^2)	Amount Stretched (μ^2)	Time To Reach Equilibrium (Min.)	Stretching Rate (μ^2/Min.)	Amount Stretched in 70 Min. (μ^2)
50	23,700	8,300	28	296	24,430
40	24,024	8,624	11	784	26,947
Percentage of increase	.....	3.9	.....	164.0	10.3

eggs were $3\frac{3}{4}$ hr. old. The osmotic pressures increased by 27, 45, and 64 per cent, respectively. The bursting rates increased about ten times more than the osmotic pressures, viz., 396, 500, and 640 per cent, respectively. Dorfman (1932) also obtained accelerated bursting with increased pressure.

When water intake was relatively slow, an increase with age accelerated bursting maximally. When water intake was faster, i.e., at higher pressure or late age, the increase in bursting was progressively less. Thus, in 45 per cent sea water, the bursting time decreased with age by 26 min. 50 sec. (Fig. 5). In 10 per cent sea water however the bursting time decreased, for the same ages, by only 1 min. 32 sec. Furthermore, as the pressure increased, the rapid change in bursting time occurred during fewer and fewer ages. A constant bursting rate was therefore reached earlier (Fig. 5). Thus, in 45, 30, 20, and 10 per cent sea water, bursting time decreased rapidly for approximately the first 23, 9, $7\frac{1}{2}$, and 5 hr., respectively.

When the osmotic pressure was low, the stretching curve was tripartite, as in the lower curves of Figure 3. With increasing pressure the equilibrium phase was progressively reduced, as in upper curves of Figure 3. At high pressures there was but a single curve.

At a given pressure, not only did stretching rate increase with age, but bursting occurred at progressively lower stretching levels. In Table IV, for example, bursting at successive ages occurred after 75, 20, and 7 min. of secondary stretching. At the last age bursting occurred $1\frac{1}{2}$ min. before the previous equilibrium level. In Table VIII,

between the ages of 1/6 and 31 hr. the membranes at equilibrium were 23,700μ^2. The membranes were stretched 8,300μ^2. After 95 min. in the solution, they stretched 9,430μ^2, i.e., to 24,830μ^2, without bursting. When 35 hr. old, they burst 2,680μ^2 to 4,345μ^2 below the previous total stretching and 1,550μ^2 to 3,215μ^2 below the previous equilibrium level.

With increasing osmotic pressure at a given age, precisely the same phenomenon occurred, namely, bursting at progressively lower stretching levels.

Hence, increased rate of water intake by age (i.e., greater permeability to water) gave rise to the same phenomena as increased osmotic pressure, namely, greater stretching rate, lower amount of stretching, and markedly increased bursting. When the stretching

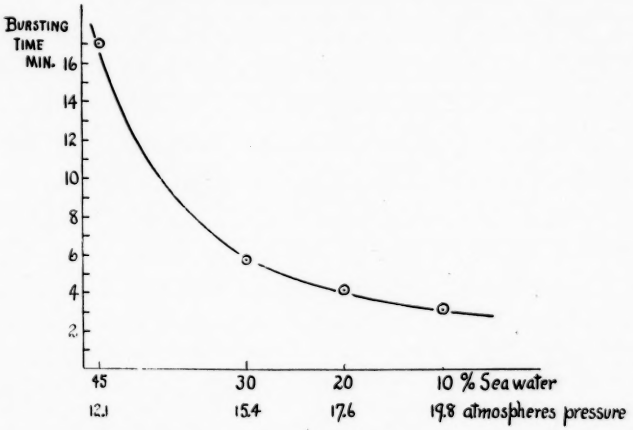

FIG. 4.—Represents bursting curve by increased osmotic pressures at a given age. This curve is similar to Figure 2. Eggs from one female, 3¾ hr. old, tested in 45, 30, 20, and 10 per cent sea water, 20.5° C.

rate was rapid, further increase, by age or by pressure, resulted in little or no greater bursting rate (Figs. 2 and 5).

Since marked increase in primary stretching rate occurred at earliest ages, when there was little or no deterioration, and also occurred in eggs of the same age at greater pressures, it is probable either that changes in tensile strength and elasticity of the membrane did not occur or that they played a minor rôle in the greater stretching and bursting rates.

INJURY

Injury by age or by hypotonic sea water was determined by reduction in percentage of cleavage. Freshly shed eggs in good condition were subjected to 60 per cent sea water at 21° C. At successive stages in the swelling curve, samples were removed to sea water and, after an adequate interval, inseminated. During primary swelling and early equilibrium cleavage was the same as in control eggs. During late equilibrium and thereafter there was progressively greater injury. Injury, therefore, was accompanied by secondary swelling. At each age thereafter injury occurred progressively earlier in the swell-

ing curve. Such earlier and greater injury was associated with progressively earlier onset of equilibrium, shorter equilibrium phase, and earlier secondary swelling.

It was previously shown (Goldforb, 1935*b*) that permeability to water increased during early ages with no detectable injury to the eggs. With age and further increase in permiability, there was progressive injury. At late ages and marked deterioration permeability increased very rapidly. Lucké (1931) and Lucké and McCutcheon (1930, 1932) also found a sharp increase in permeability after severe injury by various agents. It is therefore assumed that secondary swelling is an expression of the sharp increase in

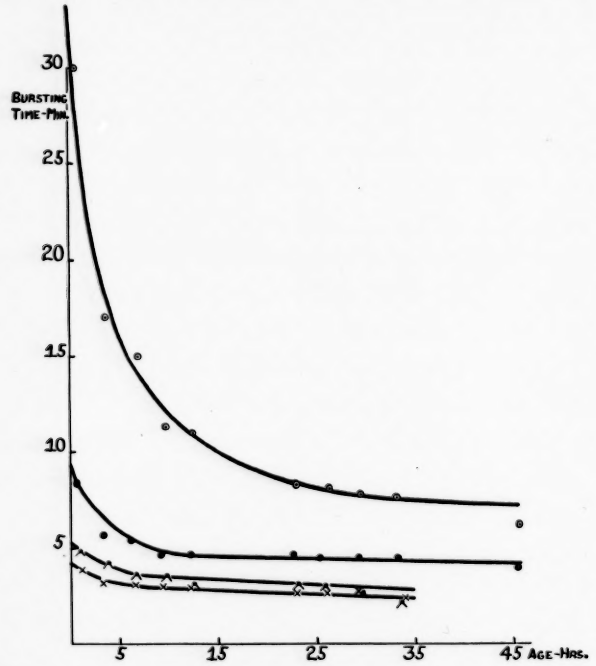

FIG. 5.—Shows effect of increasing osmotic pressure and age. The greater the pressure, the smaller the change in bursting time with age. Eggs from one female, tested at each age in 45, 30, 20, and 10 per cent sea water, at 20.5° C., phH 8.3.

⊙ represents bursting time in 45 per cent sea water; •, in 30 per cent; ∧, in 20 per cent; and ×, in 10 per cent sea water.

permeability following severe injury. Leitch (1935) observed other evidence of injury with secondary stretching, viz., vacuolization. Other factors are undoubtedly involved in secondary swelling and injury, such as changes in ionic concentration (Winterstein, 1916; Stiles and Kidd, 1919*a*, 1919*b*; Osterhout, 1922; Brooks, 1929) and, also, colloidal swelling and liquefaction of the protoplasm (Goldforb, 1935*c*).

From the foregoing considerations it may be concluded that the primary factor in increased bursting, whether by age or by greater osmotic pressure, is the rate of water

intake. This factor controls the increased rate of membrane stretching, the decreased amount of stretching, and the disproportionately faster bursting. When stretching was slow, as in freshly shed eggs in 80 per cent sea water, some eggs in each sample died without bursting. As the stretching rate increased, either by age or by greater osmotic pressure, not only did all eggs burst but they did so more explosively. Rate of water intake, therefore, and not injury per se, determined bursting.

TABLE VIII

EXPERIMENT R: AT LATE AGE EGGS BURST AT MUCH LOWER STRETCHING LEVELS*
(Measured in 50 per Cent Sea Water at 20.5° C.)

Membrane Size at Bursting (35 hr.)	Amount Stretched (35 hr.)	Minutes To Burst (35 hr.)	Minutes To Stretch Same Amount at Preceding Ages		
			31 Hr.	6 Hr.	⅛ Hr.
μ^2	μ^2				
23,070†.....	6,430	†	14.0	13.7	17.0
22,150.....	5,510	6.5	7.0	8.0	10.0
21,755.....	5,115	7.0	6.8	7.5	9.1
21,450.....	4,810	5.0	5.3	6.0	8.3
21,365.....	4,725	5.0	5.3	6.1	8.1
21,110.....	4,470	4.0	4.8	5.3	7.4
20,485.....	3,845	4.3	4.2	4.7	5.7

* At previous ages membranes at equilibrium were 23,700 μ^2; amount stretched, 8,300 μ^2. After 95 min., membranes were 24,830 μ^2; amount stretched, 9,430 μ^2; no eggs burst.

† This egg reached equilibrium in 9 min., remained in equilibrium for 4 min., when measurements were terminated.

CONCLUSIONS

1. Freshly shed eggs, from different females, subjected to the same dilution of sea water, temperature, and pH, burst at rates which varied by about 100 per cent.

2. Eggs from each female burst progressively faster with age. The increase was over 500 per cent.

3. Bursting increased maximally during earliest ages, i.e., when least deteriorated, and changed minimally at latest ages. Increased bursting, therefore, was not determined by deterioration per se.

4. Increased bursting with age was correlated with progressively greater stretching rate of the plasma membrane.

5*a*) In 45–80 per cent sea water, the curve of stretching was tripartite, viz., primary stretching, equilibrium, and subsequent secondary stretching.

b) The amount of primary and of total stretching did not increase significantly with age.

c) Primary stretching rate increased with age by from 126 to 160 per cent. Secondary stretching rate also increased by 29–100 per cent.

d) Equilibrium occurred more quickly, and its duration was progressively shorter, with age.

e) Secondary stretching began more quickly in older eggs.

6*a*) Increased stretching rate with age was due to increasing permeability to water.

b) Increased stretching rate resulted in correspondingly decreased amount of total stretching.

c) Hence the disproportionately faster bursting with age.

d) When water intake was slow, an increase, during early ages, resulted in marked increase in stretching rate and maximally increased bursting. When water intake was rapid, i.e., at late ages, the increase in stretching and bursting rates was minimal.

7. Injury was followed by secondary stretching.

8. Precisely the same phenomena (items 4–7, inc.) occurred in eggs of a given age (and therefore with membranes of the same tensile strength and elasticity) by a small increase in osmotic pressure.

9. Hence, the rate of water intake, whether by age or by greater osmotic pressure, is the chief factor in the increased stretching rate, the decreased amount of stretching, and the disproportionately greater bursting rate.

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THE COMBINED EFFECTS OF HEAVY WATER AND TEMPERATURE ON THE HEART-RATE OF THE TURTLE

(Twelve figures)

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A PREVIOUS note (Barnes and Warren, 1935) reported that heavy water reduces the rate of pulsation of the excised heart of the frog. The present paper deals with a similar effect on the auricles of the turtle, which were found to survive longer under the experimental conditions imposed by a limited quantity of fluid for immersion. In view of the similarity of the effects of concentrated heavy water on physiological processes with those produced by a drop in temperature, it was thought that a study of the combined effects of heavy water and temperature would throw light on the mode of action of the heavy hydrogen isotope on living systems. Brandt and Reindell (1936) have confirmed our work on the frog's heart and also find disturbances of the electrocardiogram in heavy water, but they did not control the temperature.

The general biological effects of heavy water have been reviewed elsewhere (Barnes and Jahn, 1934; Barnes, 1935). Most investigators who have used concentrated heavy water report either a retardation of physiological processes or, in a few cases, no obvious effect. Fox and Craig (1935) on the other hand claim that the activity of enzyme extracts is sometimes actually accelerated by concentrated heavy water. Inasmuch as this accelerating effect of concentrated heavy water has not been observed in living protoplasm, it is probably a secondary effect which involves the gradual destruction of the enzymes. Our colleague, Mr. E. J. Larson, will publish shortly on these enzyme reactions. The influence of heavy water in the modification of normal physiological processes can be studied in low concentrations in the organism as a whole (Barnes and Larson, 1934). However, the short survival and the inherent variation in the rate of the heart (cf. Loomis and Harvey, 1930) forced us to use relatively high concentrations of deuterium oxide.

METHODS

Auricles with sinus attached were excised and suspended by a thread passing through both auricles in a test tube containing 2 cc. of oxygenated Ringer's solution immersed in a thermostat. Fourteen preparations were studied, chiefly from *Pseudemys troostii*. A few hearts from *P. scripta* were also observed (indicated in the figures). Two cubic centimeters of Ringer's solution buffered with phosphate to pH 8.2 were evaporated on a steam bath and redissolved in 2 cc. of ordinary distilled water or in 2 cc. of heavy water. Seven hearts were run in heavy water, and seven served as controls. After a preparation ceased beating or became very feeble, it was transferred to 2 cc. of ordinary Ringer's solution which had been adjusted to the required temperature. The time for 10 beats was taken with a stop watch at intervals of 1°. The temperature was constant to within 0.2° as read on a certified thermometer.

The Arrhenius equation

$$ln \frac{R_2}{R_1} = \frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

where R_1 is the rate at T_1 and R_2 is the rate at T_2, was applied to the data representing the frequency of the beat as controlled by temperature. The constant μ is "the temperature charasteristic" or energy of activation of the pacemaker expressed in calories per gram molecule (cf. Crozier, 1926).

The 2 cc. of heavy water in the Ringer's solution was undoubtedly diluted when the auricles with blood or Ringer's solution were introduced. The volume of the preparations, together with contained fluid, was 0.6–0.8 cc., which would reduce the percentage of heavy water by about one-fifth. We do not know the percentage of heavy water attained in the contractile mechanism of the heart. Lucké and Harvey (1935) find that heavy water enters the sea-urchin egg at the same rate as ordinary water, but Parpart (1935) reports that heavy water enters the erythrocyte at almost half the normal rate.

INOTROPIC, CHRONOTROPIC, AND SURVIVAL EFFECTS

As no data were available concerning the normal behavior of the turtle auricles in only 2 cc. of Ringer's solution, seven controls were run. Figure 1 shows that a preparation may continue beating for 6 hours with one change of solution. In this experiment the heart was finally arrested by high temperature (about 40° C.).

The first effect of the heavy water was a reduction in the amplitude of the beat (as in some forms of vagus inhibition), followed by a decrease in frequency. For the study of the kinetics of temperature effects only *rates* of reaction can be used; so this report deals only with the chronotropic effect.

There is considerable variation in the normal rate of different preparations, but the average rate at each temperature is lower in the heavy water (D_2O Ringer) (Fig. 2). Thus, at 10° C. the average rate of five hearts was 9.7 beats per minute in H_2O Ringer, and the average rate for four hearts in Ringer containing 80–96 per cent D_2O was 6.1 beats per minute. As pointed out above, the heavy water was probably diluted by one-fifth on introduction of the hearts. At 20° C. the average rate of five hearts in H_2O Ringer was 28 beats per minute, and the rate for four hearts in 80–95 per cent D_2O Ringer was 21.2 beats per minute. That the reduced rate in heavy water was not a result of slowing with age of the preparation is indicated by the following average ages of the hearts represented:

At 10° C.: H_2O, 45.6 min.; D_2O, 35.5 min.
At 15° C.: H_2O, 54.2 min.; D_2O, 45.8 min.
At 20° C.: H_2O, 26.6 min.; D_2O, 23.5 min.

Besides the inotropic and chronotropic effects, it was observed that the preparations in heavy water did not survive so long as the controls. Thus the average time elapsing before standstill was only 86.1 minutes in D_2O Ringer, while the average longevity of the control hearts was more than 126 minutes. With the latter preparations we did not continue observations until standstill in all cases.

It is necessary to distinguish clearly between the negative inotropic and chronotropic effects of heavy water and (1) the weakening of the preparation with time, (2) the effect

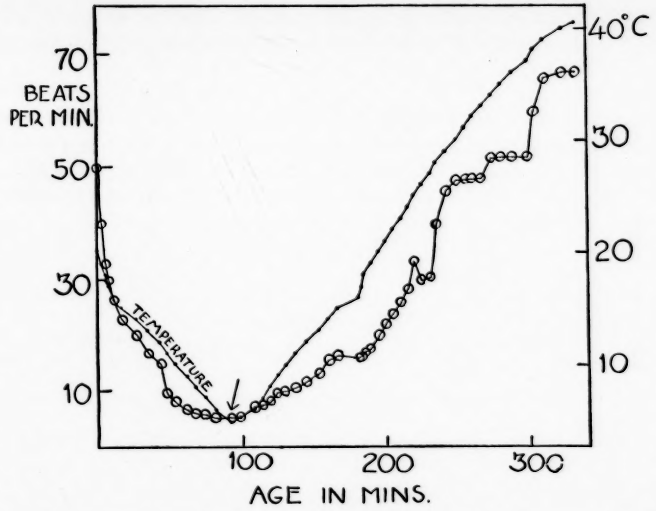

FIG. 1.—Beating of the turtle auricles in 2 cc. of ordinary Ringer's solution (oxygenated) as controlled by temperature. ○, beats per minute (ordinates at left); ●, temperature in degrees centigrade (ordinates at right); abscissas, age of preparation in minutes. After 95 minutes (indicated by arrow) the preparation was transferred to 2 cc. of fresh Ringer's solution. Above 20° C. the amplitude of the beat was very weak. Before the experiment the auricles were washed in Ringer's solution.

Pseudemys troostii, No. 77, November 12. Turtle from tank at 20 °C.

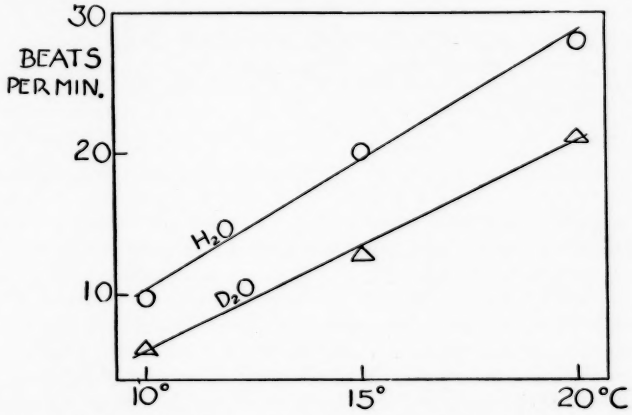

FIG. 2.—Average rates of turtle auricles in 2 cc. of ordinary Ringer solution and Ringer's solution containing 80–96 per cent heavy water at 10°, 15°, and 20° C. ○, H_2O Ringer; △, D_2O Ringer. Each point is the average of from four to six hearts.

of changing the Ringer's solution, (3) the effect of temperature, and (4) the natural difference in the rates of pulsation of different preparations. By suitable experimental procedure these separate factors can be demonstrated.

In one experiment (Fig. 3) the temperature was kept constant at 16.8° C., and two preparations were observed simultaneously in H_2O Ringer and in 86 per cent D_2O Ringer. The auricles placed in the heavy water had a faster rate than the controls before the experiment but were arrested by the heavy water within 100 minutes. Both preparations were then transferred to fresh H_2O Ringer, and immediate recovery from the deuterium standstill occurred. After 188 minutes (from beginning of experiment) (not shown in

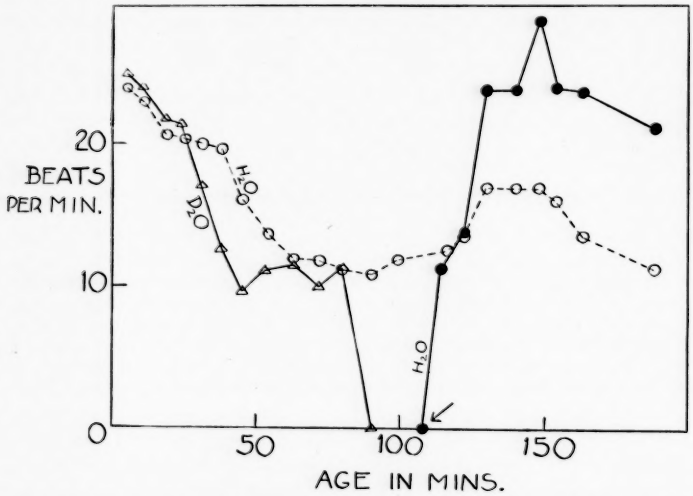

FIG. 3.—Two preparations observed simultaneously at constant temperature (16.8° C.). ○, auricles in H_2O Ringer; △, another preparation in 86 per cent D_2O Ringer. At the point marked by the arrow each preparation was transferred to 2 cc. of fresh Ringer (at 16.8° C.). ●, beat of the heavy-water heart after transfer to H_2O Ringer.

Pseudemys scripta, No. 86, November 27. Turtle from tank at 18° C.

Fig. 3) the original heavy-water heart which had recovered in the fresh H_2O Ringer was transferred to the used sample of H_2O Ringer, in which it continued to beat without appreciable change in rate. At the same time the control heart was transferred to the used sample of D_2O Ringer, in which the heart stopped. After 25 minutes, the original heavy-water heart beating in the used sample of H_2O Ringer was returned to the original sample of D_2O Ringer, which arrested the beat. Five minutes later it was revived by transfer to the used H_2O Ringer solution. Hence, in this experiment the inhibiting influence of heavy water was shown independently of temperature change, age of preparation, difference in initial rate, and influence of renewed Ringer's solution.

The combined depressing effects of heavy water, drop in temperature, and aging of preparation may be seen in Figure 4; but recovery on transfer to ordinary Ringer's solution is quite evident. As in most preparations, the recovery beat after deuterium standstill is immediate and indicates that there is no irreversible toxic action on the heart.

The similar effect of temperature in both D_2O Ringer and H_2O Ringer is seen in Figure 5. In this preparation there was no chronotropic effect of heavy water at first, but sudden slowing occurred after 40 minutes in D_2O Ringer.

TEMPERATURE CHARACTERISTICS OF THE HEART-RATE

The special conditions imposed by only 2 cc. of solution did not seriously disturb the regularity of the effects of temperature in all the preparations, but it was thought necessary to obtain temperature characteristics in 2 cc. of ordinary Ringer's solution as a

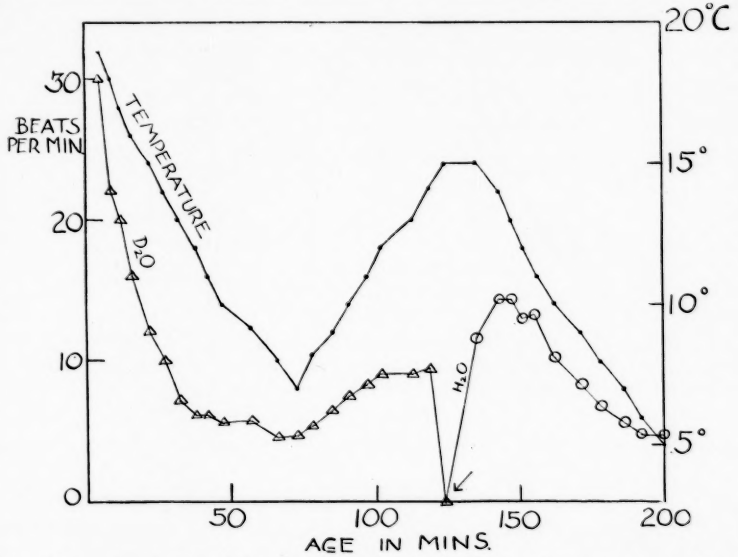

FIG. 4.—The combined effects of heavy water and temperature on the heart-rate. △, beats per minute in 80 per cent D_2O Ringer; ○, rate after transfer to H_2O Ringer (ordinates at left); ●, temperature in degrees centigrade (ordinates at right). Note the rapid fall in rate produced by the similar action of heavy water and falling temperature. At the point marked by the arrow the preparation was transferred to H_2O Ringer.

Pseudemys troostii, No. 75, November 11. Turtle from tank at 17° C. See Figure 11.

basis for comparison with the temperature graphs representing hearts in D_2O Ringer. The possibility of obtaining satisfactory temperature graphs from these preparations is illustrated in Figure 6, which shows a remarkably regular rectilinear relation between the log of the heart-rate and the reciprocal of the absolute temperature as required by the Arrhenius equation. This control heart gave a μ value of 24,700 calories in the first run from 19° to 4° C., which is the entire temperature range that can safely be applied to the turtle heart (cf. Markowski, 1933). After reaching 4° C. the temperature was then raised and the log of the heart-rate followed the same line to 8.4° C. and then gave a new slope (μ = 8,000 calories) to 14° C. From 14° C. to 20° C. the slope changed again, giving a μ-value of 17,200 calories. The irregular nature of this run was probably a result of the accumulation of metabolites in the Ringer's solution. After changing the

Ringer's solution, an even line was obtained from 5° to 16° C., giving a μ-value of 23,400 calories. Above 16° C. the temperature characteristic changed to 16,700 calories (see Table I).

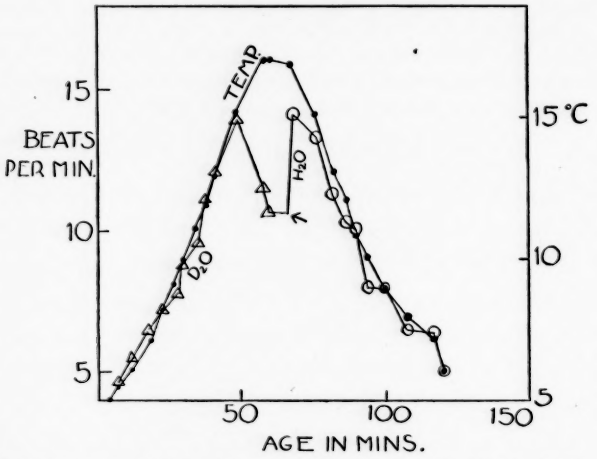

FIG. 5.—Delayed chronotropic effect of 80 per cent D_2O Ringer. Symbols as in Figure 4. Note how closely the heart-rate follows the temperature change both in D_2O Ringer and H_2O Ringer.
Pseudemys troostii, No. 44, May 31. Turtle from tank at 20° C. See Figure 9.

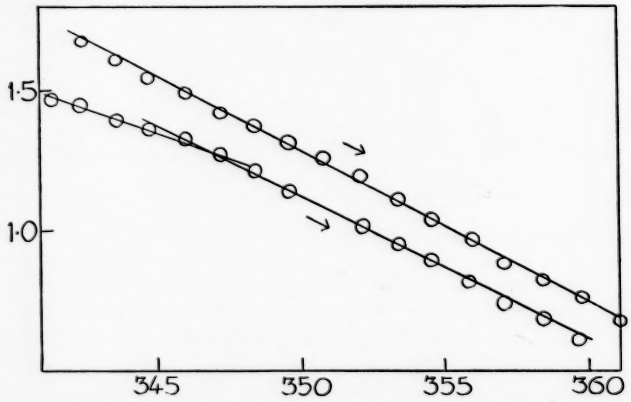

FIG. 6.—Relation between the log of the rate of pulsation (ordinates) of turtle auricles in 2 cc. of H_2O Ringer and the reciprocal of the absolute temperature ($\times 10^5$) (abscissas). The upper line represents the first run, which gave a temperature characteristic of 24,700 calories. The lower line represents the heart-rate after transfer to fresh H_2O Ringer; $\mu = 23{,}400$ calories below 15° C. and 16,700 calories above 15° C. A second run in the first H_2O Ringer sample gave the following μ-values: 24,700 (4°–8.5° C.), 8,000 (8.5°–12.4° C.), 17,200 (12.4°–20° C.).
Pseudemys scripta, No. 80, November 17. Turtle from tank at 12° C.

The temperature characteristics were not the same in each preparation of the controls in H_2O Ringer. Figure 7 represents a heart which gave a μ-value of 19,300 calories for two runs in two samples of H_2O Ringer. Figure 8 shows a heart in H_2O Ringer which

TABLE I

TEMPERATURE CHARACTERISTICS FOR TURTLE AURICLES, ARRANGED IN THE ORDER OF THE EXPERIMENTS

μ-values in parentheses are not well established, owing to scatter of points on the graph. Hearts of *Pseudemys troostii*, except Nos. 80 and 84, which are from *P. scripta*.

Expt. No.	Fig. No.	Date 1935	Medium	Temperature Characteristic (Calories)	Temperature Range (Degrees Centigrade)	Remarks
39	12	May	D_2O, 50%	19,550	5–14	.....
39	12	May	D_2O, 50%	12,200	14–20	.....
44	5, 9	May	D_2O, 80%	16,600	4.8–17.1	.....
44	5, 9	May	H_2O	16,600	16.9–4.9	Fresh H_2O, after D_2O
49	8	June	H_2O	20,500	5–13	.....
49	8	June	H_2O	11,500	13–21	.....
49	8	June	H_2O	(20,500)	8–14	Fresh H_2O
55	7	June	H_2O	19,300	2–26	.....
55	7	June	H_2O	19,300	7–16	Fresh H_2O
63	10	June	D_2O, 96%	16,700	5–20	.....
63	10	June	H_2O	28,000	5–9	Fresh H_2O, after D_2O
63	10	June	H_2O	16,200	9–21	Same Ringer as above
71	.....	Nov.	D_2O, 85%	(14,450)	4–15	Wide scatter of points
72	.....	Nov.	H_2O	.....	4–14	Irregular
74	.....	Nov.	D_2O, 95%	(14,500)	14–19	Only 5 points
74	.....	Nov.	H_2O	(22,300)	2–12	Wide scatter of points
75	4, 11	Nov.	D_2O, 80%	33,900	19–12	.....
75	4, 11	Nov.	H_2O	26,100	13–4	.....
77	1	Nov.	H_2O	22,700	19–12	.....
80	6	Nov.	H_2O	24,700	19–4	.....
80	6	Nov.	H_2O	23,400	4–15	Fresh Ringer
80	6	Nov.	H_2O	16,700	15–20	Fresh Ringer
80	6	Nov.	H_2O	24,700	4–8.5	Back in original Ringer
80	6	Nov.	H_2O	8,000	8.5–12.4	Same
80	6	Nov.	H_2O	17,200	12.4–20	Same
84	.....	Nov.	H_2O	(23,300)	4–8	Only 4 points
84	.....	Nov.	H_2O	(12,937)	8–13	Same
84	.....	Nov.	H_2O	25,300	7–20	Fresh Ringer
88	.....	Nov.	H_2O	.....	.....	Too irregular for analysis

gave a μ-value of 20,500 calories between 3.1° and 13° C. and a different value of μ, i.e., 11,500 calories above 13° C. In a second run in fresh H_2O Ringer the original μ-value of 20,500 calories appeared (8.2°–14° C.); but the points on the graph do not fall directly on the line, as is often the case with data representing the rate of old preparations.

That heavy water does not necessarily change the temperature characteristic is seen in Figure 9. This heart gave a μ-value of 16,600 calories in 80 per cent D_2O Ringer and the same value when transferred to H_2O Ringer. It will be seen in Figure 9 that in this

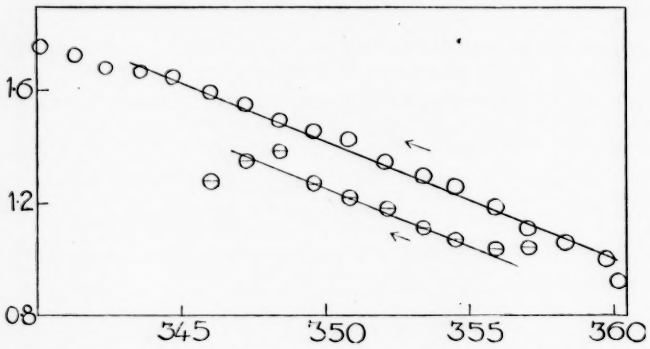

FIG. 7.—Preparation in H_2O Ringer (ordinates and abscissas as in Fig. 6) with a temperature characteristic of 19,300 calories. The lower graph (barred circles) was obtained after transfer to 2 cc. of fresh H_2O Ringer.
Pseudemys troostii, No. 55, June 14. Turtle from tank at 20° C.

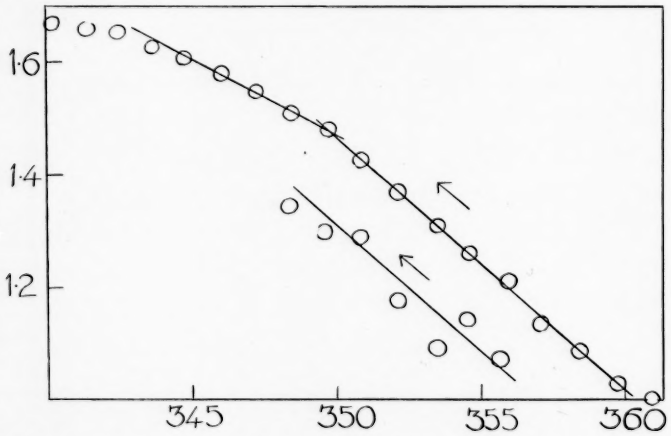

FIG. 8.—Preparation giving two temperature characteristics in H_2O Ringer, 20,500 calories below 13° C. and 11,500 calories above 13° C. Symbols as in other figures. Lower line represents a second run in fresh H_2O Ringer, $\mu = 20{,}500$ calories (8.2°–14° C.).
Pseudemys troostii, No. 49, June 12. Turtle from tank at 20° C.

preparation the negative chronotropic effect of deuterium was considerably delayed. A similar effect is recorded in Figure 10. Here the μ-value was 16,700 calories in 96 per cent D_2O Ringer. On transfer to H_2O Ringer the first μ-value was 28,000 calories (from 5° to

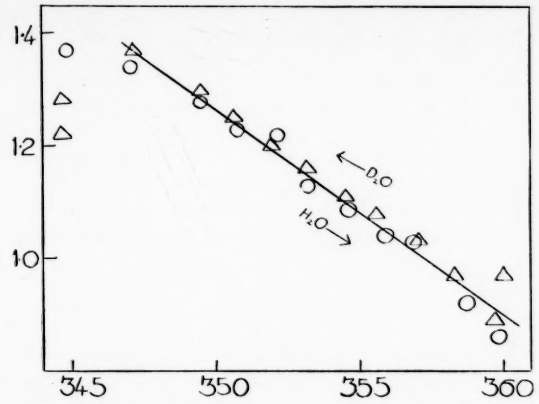

FIG. 9.—A preparation which gave the same temperature characteristic, 16,600 calories, in both H_2O Ringer and 80 per cent D_2O Ringer (ordinates and abscissas as in Fig. 7). ○, H_2O Ringer; △, D_2O Ringer. The first run was made in D_2O Ringer. The chronotropic effect of deuterium did not appear until the end of the run.

Pseudemys troostii, No. 44, May 21. Turtle from tank at 20° C. See Figure 5.

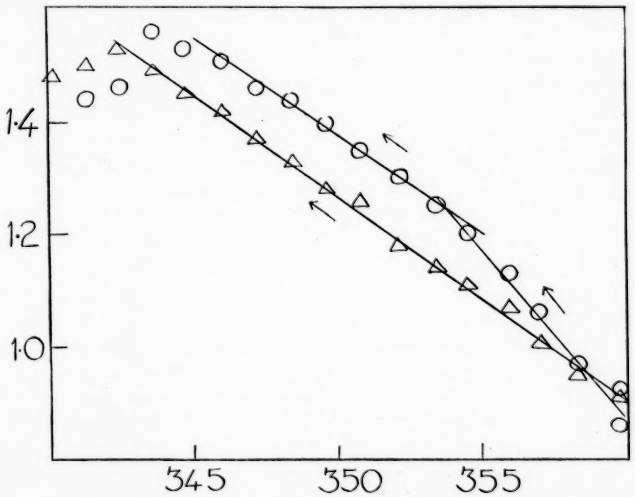

FIG. 10.—A preparation which gave a temperature characteristic of 16,700 calories in 96 per cent D_2O Ringer and 16,230 calories in H_2O Ringer. Symbols as in Figure 8. The lower line (△ = D_2O) represents the first run. Then the auricles were transferred to H_2O Ringer (upper line, ○). Between 5° and 9° C. the recovery slope is steep, giving a μ-value of 28,000 calories. From 10° to 20° C. the slope is almost parallel to the former line for the heart in D_2O Ringer.

Pseudemys troostii, No. 63, June 18. Turtle from tank at 20° C.

9° C.), after which the slope of the line is almost parallel to the line representing the rate in heavy water. Figure 11 represents a preparation giving a temperature characteristic

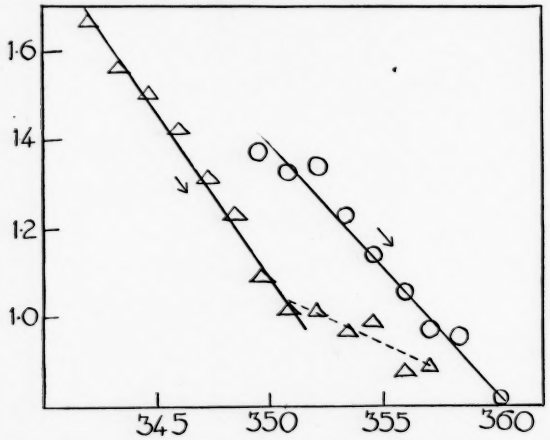

FIG. 11.—A preparation which gave a temperature characteristic of 33,900 calories in 80 per cent D_2O Ringer (line through triangles) (symbols as in other figures). After transfer to H_2O Ringer, $\mu = 26{,}100$ calories (line through circles).

Pseudemys troostii, No. 75, November 11. Turtle from tank at 15° C. See Figure 4.

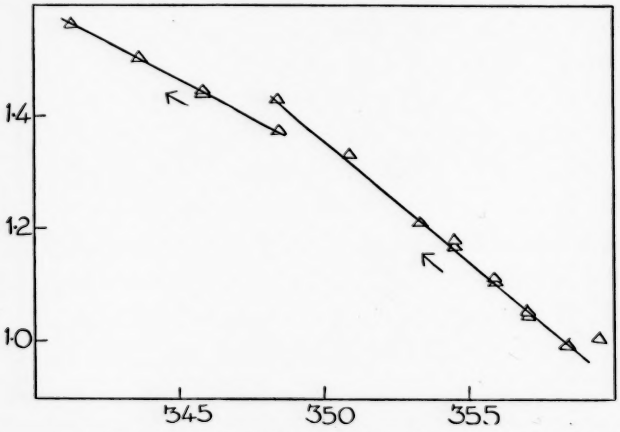

FIG. 12.—A preparation in 50 per cent D_2O Ringer giving two temperature characteristics, 12,200 calories above 14° C. and 19,550 below 14° C. Symbols as in other figures.

Pseudemys troostii, No. 39, May 12. Turtle from tank at 20° C.

of 34,000 calories in 80 per cent D_2O Ringer and 26,000 calories after transfer to H_2O Ringer. A preparation in 50 per cent D_2O Ringer (Fig. 12) yielded two values of μ:

12,200 calories between 14° and 20° C. and 19,500 calories in the temperature range of 5° and 14° C.

The temperature characteristics reported above were obtained from graphs in which the log of the frequency of the heart-rate showed a regular rectilinear relation when plotted against the reciprocal of the absolute temperature as required by the Arrhenius equation. In two preparations, however, one in 86 per cent D_2O and one control, no satisfactory readings were obtained. In four other hearts (indicated in Table I) the plotted points for some of the runs showed quite a wide scatter from the hypothetical straight lines, and consequently these values are not accepted as final in the following discussion of the theoretical implications of the experimental results.

DISCUSSION

The effect of heavy water on the heart-rate is in many respects similar to a drop in temperature. The lower energy content of heavy water is clearly indicated by its higher freezing and boiling points and its reduced molecular vibrations (Bernal and Tamm, 1935). That the slowing of the beat is largely a matter of reduction in energy rather than some "toxic" action during short exposure is demonstrated by the striking recovery on return to ordinary water. The unique properties of heavy water make it possible to investigate the effects of reduced energy without change in temperature. This affords many interesting possibilities in the physiology of warm-blooded animals. Barbour (1935), for example, has reported that heavy water produced a striking reduction in the basal metabolism of the mouse.

In one respect the heavy-water effect does not parallel that of a drop in temperature, in that the preparations do not survive as long in D_2O Ringer.

That heavy water does not produce profound chemical changes in the heart is also inferred from the study of temperature characteristics or energies of activation. According to Urey and Teal (1935), a change in the energy of activation of a chemical process taking place in heavy water need not be expected if the reaction does not directly involve hydrogen. If heavy hydrogen is directly involved (as in the reaction $Br + D = DBr$), the energy of activation will be higher to the extent of the difference in the zero-point energy of the two isotopes (approximately 1,500 calories) (Melville and Rideal, 1935). This simple explanation cannot be applied to the temperature characteristics of hearts in heavy water.

The μ-values obtained are distributed in the vicinity of modes 11,000, 16,000, 20,000, and 24,000 calories, which occur as master reactions in many diverse physiological phenomena (Crozier and Hoagland, 1934). In a given preparation, immersion in heavy water does not necessarily change the master reaction. Crozier (1926) has pointed out that the value for frog heart-beat frequency may differ independently of deliberate experimental treatment (which illustrates the independence of the heart as a spontaneously rhythmic mechanism). Also, the rate of the frog's heart beat can be increased by adrenaline without change in the temperature characteristic (Crozier, 1926).

The μ-value of 16,000 calories is associated with iron catalysis of oxidation (Crozier, 1924)—a reaction which might proceed at a lower rate in heavy water owing to the reduced energy but would not be fundamentally altered by the presence of the hydrogen isotope.

If the effect of heavy water is equivalent to a drop in temperature, no change of the

temperature characteristic need occur, for the slope of the line in the temperature graphs for heart rates is often the same at high and at low temperatures.

Snyder's (1905) data for the rate of the excised whole heart of the terrapin (in moist air) give a μ-value of 13,000 calories. This value was not a common one under the conditions of our experiments, but similar values occurred in one control (12,900 calories) and also in a heart in 50 per cent D_2O (12,200 calories). (Fig. 12). The value of 16,000 calories, common for heart-rates, is also characteristic of heat liberation of frog skeletal muscle (Crozier, 1926). This does not necessarily imply a myogenic pacemaker; for, as Glaser (1929) has pointed out, the mechanism of nervous control might well have a master reaction identical with a myogenic pacemaker which is suggested by the similar metabolism of muscle and nerve. If Heinbecker and Bishop's (1935) recent revival of the theory of a neurogenic pacemaker in the turtle heart is accepted, the heavy water apparently reduced the duration and then the frequency of discharges from ganglia.

SUMMARY

1. The average rate of pulsation of turtle auricles is slower in 2 cc. of oxygenated Ringer's solution containing 50–96 per cent heavy water than in controls under the same conditions.

2. The effect of heavy water (approximately 75 per cent) on the heart-rate is equivalent to a drop in temperature of about 5° C.

3. The amplitude of the auricular beat is depressed in heavy water, and standstill occurs much sooner than in controls.

4. In the majority of auricular preparations in only 2 cc. of Ringer's solution containing heavy or ordinary water, the log of the rate of pulsation shows a very regular rectilinear relation with the reciprocal of the absolute temperature as required by the Arrhenius equation.

5. A given preparation under the conditions of the experiment may exhibit one or more of several temperature characteristics, but there is no satisfactory evidence that heavy water necessarily changes the master reaction or pacemaker when it reduces the rate of pulsation.

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A QUANTITATIVE ANALYSIS OF THE PITUITARY-OVULATION RELATION IN THE FROG (RANA PIPIENS)

(Seven figures)

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THE variations normally experienced in the pituitary-ovulation experiments on the frog, *Rana pipiens*, indicate the need for more accurate quantitative data. Even with the standard dose of two adult female pituitaries, the frogs do not always give a reaction, nor an identical reaction in any two cases. It has previously been shown (Rugh, 1935) that the degree to which the ovaries of a particular frog are emptied of eggs depends upon the amount of anterior pituitary hormone injected. Obviously, then, there are several possibilities to be investigated, i.e., either the dose of injected anterior pituitary varies quantitatively or the recipient shows variations in susceptibility to the hormone or both variables may be involved.

With these possibilities in mind, an experiment was planned so as to determine first any correlation between the factors of sex, body weight, body length, gonad weight, and anterior pituitary weight. In the second part of the experiment it was planned to determine the anterior pituitary potency per unit of weight of the gland, from both males and females, as measured by the degree of ovulation. In order to include possible seasonal variations, one set of data was collected in November and another in February. Females of the species *R. pipiens* cannot readily be induced to ovulate much before September, probably because the accumulation of egg yolk has not, by that time, reached its "maturity" level. These frogs normally ovulate between April 1 and May 15; hence February was chosen as the latest non-ovulation period. November and February represent the first and the last of the normal hibernation period.

During November, 120 females and 140 males were used; and during February, 119 females and 140 males were used. These frogs were secured from the same source; and, according to Table I, the two groups showed remarkable uniformity.

METHOD

The experiment was so timed that the frogs would be used within 1 week after receipt at the laboratory, direct from their natural environment in Vermont. Upon receipt, the frogs were segregated into five groups, depending upon size. The measurements were taken from the tip of the snout to the opening of the cloaca (anus); and the groupings were as follows: those less than 75 mm., those 75–80 mm., 80–85 mm., 85–90 mm., and those with body length greater than 90 mm. Each frog was numbered, pitched, measured for body length to millimeters, and weighed; the gonads were removed and weighed; and the anterior pituitary was removed, freed from all attached tissues, and placed in a 2-cc.

[1] The author wishes to express here his sincere appreciation for the facilities offered by the Department of Zoölogy, Columbia University, which made this work possible. Particularly grateful is he for the cooperation freely given by Professor L. G. Barth, for the tireless assistance of Mr. John A. Moore in the collection of data, and for the technical assistance of Mr. Jack Godrich.

homeopathic vial, which was immediately corked. All vials were numbered and very carefully weighed to the fourth decimal. Each vial was to contain the anterior pituitaries from twenty frogs of the same sex and approximately the same body length. It was found that individual anterior pituitaries weighed from 0.5 to 1.3 mg.; hence to reduce observational error it was decided to weigh groups of twenty glands from similar frogs. After the weight of the glands had been determined, they were transferred to a larger vial containing as many cubic centimeters of distilled water as there were milligrams of gland tissue. In this way, with maceration, the suspension was equivalent to 1 mg. of anterior pituitary per cubic centimeter of water. The vial was then placed in the refrigerator at 2°–4° C. until it was used, generally within 24 hours.

The second part of the experiment, i.e., that dealing with the ovulation reactions elicited by quantitatively determined doses of the anterior pituitary, was carried on with

TABLE I

SIZE RELATIONS OF FROGS IN NOVEMBER AND FEBRUARY

Sex	Number Used	Season	Average Length (Mm.)	Average Body Weight (Gm.)	Average Gonad Weight (Gm.)
Males	140	November	75.10	35.94	.....
Males	140	February	75.77	34.37	.....
Females	120	November	76.67	43.60	7.83
Females	119	February	78.88	39.86	6.57

females showing the same size variations as the donors listed above. Forty-five females were used in November, and 26 in February. Some of these females were used to test the potency of the pituitaries taken from frogs after induced ovulation. In each case the percentage of ovulation was determined by weighing the eggs which had been liberated from the ovaries, whether in the uteri or body cavity, and weighing the ovary containing eggs which had not been freed from their follicles. Ample time was allowed, so that ovulation had been completed before these examinations were made.

EXPERIMENTAL DATA

The quantitative data in Tables II, III, IV, and V represent the averages of twenty readings in each case. These were the donors of the anterior pituitary hormone which was used in the second part of the experiment pertaining to ovulation.

The data in Tables VI and VII pertain to the ovulation reaction, in frogs of various body lengths, to varying doses of anterior pituitary gland from the sources listed in Tables II–V. The data are given in full because they not only show individual variations in reactions to equivalent doses but also show clearly the importance of the source of the hormone. Under the heading "Source of Pituitary" is given the sex and average body size of the donors. The body size represents the average of the twenty donors.

Tables VI and VII represent the data collected in November and February, while Table VIII gives data on the potency of the pituitaries removed from females which had previously been induced to ovulate. The potency is measured not only by the degree of ovulation but by the ability to induce amplexus in the male.

TABLE II

Analysis of female frogs in November

Length (Mm.)	Total Weight (Gm.)	Gonad Weight (Gm.)	Anterior Pituitary Weight (Mg.)
75.2	38.2	6.87	0.860
78.0	38.4	7.52	0.972
78.6	41.6	7.08	0.995
79.45	44.5	7.79	1.040
81.3	47.2	8.40	1.145
83.5	51.6	9.34	1.190

TABLE III

Analysis of female frogs in February

Length (Mm.)	Total Weight (Gm.)	Gonad Weight (Gm.)	Anterior Pituitary Weight (Mg.)
71.66	27.80	3.13	0.626
76.47	34.50	5.35	0.735
77.00	34.85	5.52	0.800
82.50	44.45	8.01	1.110
86.79	53.73	10.85	1.395

TABLE IV

Analysis of male frogs in November

Length (Mm.)	Total Weight (Gm.)	Gonad Weight (Gm.)	Anterior Pituitary Weight (Mg.)
71.3	31.6	0.80	0.615
73.2	32.1	1.20	0.638
74.4	36.6	1.30	0.850
75.2	36.0	1.40	0.915
76.6	37.9	1.00	1.130
77.2	37.4	0.70	1.290
77.8	40.0	1.40	1.520

TABLE V

Analysis of male frogs in February

Length (Mm.)	Total Weight (Gm.)	Anterior Pituitary Weight (Mg.)
71.40	27.85	0.645
72.95	28.17	0.650
75.35	33.82	0.895
75.55	34.05	0.920
75.70	35.77	0.930
78.45	37.75	1.040
81.05	43.20	1.175

TABLE VI

ANTERIOR PITUITARY INDUCED OVULATION IN NOVEMBER

No.	Body Length (Mm.)	Body Weight (Gm.)	Source of Pituitary		Weight of Pituitary (Mg.)	Total Ovarian Weight (Gm.)	Percentage of Ovulation
			Sex	Length (Mm.)			
1	92.0	57.0	female	75.2	2	18.7	0.0
2	91.0	62.0	female	75.2	2	11.5	0.0
6	97.0	83.0	female	75.2	3	17.3	0.9
11	98.0	70.0	female	78.6	4	17.0	29.0
16	92.0	63.0	female	79.4	5	19.0	95.0
101	94.0	76.0	male	71.3	2	16.0	0.0
111	93.0	63.0	male	77.2	4	10.1	1.0
116	94.0	65.0	male	76.6	5	12.0	0.0
121	92.0	63.0	male	77.8	6	25.0	88.0
131	91.0	70.0	male	74.2	8	15.1	2.0
7	88.0	56.0	female	75.2	3	13.0	0.0
12	89.0	57.0	female	78.6	4	19.1	47.0
17	89.0	57.0	female	81.5	5	24.5	95.0
103	89.0	57.0	male	71.3	2	11.0	0.0
112	88.0	59.0	male	77.2	4	12.0	8.0
117	89.0	60.0	male	76.6	5	20.0	55.0
118	87.0	57.0	male	76.6	5	12.0	0.0
122	88.0	61.0	male	77.8	6	12.0	0.0
132	88.0	63.0	male	74.2	8	23.0	87.0
134	89.0	66.0	male	74.2	8	12.4	59.0
3	82.0	57.0	female	75.2	2	11.0	0.0
4	83.0	50.0	female	75.2	2	10.8	0.0
8	83.0	53.0	female	75.2	3	14.8	4.0
9	83.0	55.0	female	78.6	3	19.0	90.0
13	82.0	56.0	female	78.6	4	13.0	0.0
14	82.0	43.0	female	79.5	4	15.5	95.0
18	84.0	41.0	female	83.5	5	17.0	95.0
19	83.0	41.0	female	83.5	5	16.2	75.0
102	84.0	41.0	male	71.3	2	17.5	0.0
104	83.0	52.0	male	71.3	2	11.5	0.0
113	83.0	59.0	male	77.2	4	8.0	0.0
114	81.0	45.0	male	77.2	4	10.8	30.5
119	81.0	43.0	male	76.6	5	7.0	0.0
123	83.0	61.0	male	77.8	6	13.0	0.0
133	83.0	70.0	male	74.2	8	14.1	1.0
5	77.0	40.0	female	75.2	2	8.0	0.0
15	77.0	50.0	female	79.5	4	10.0	0.0
20	77.0	41.0	female	83.5	5	11.0	85.0
115	78.0	41.0	male	77.2	4	6.5	0.0
120	79.0	37.0	male	75.0	5	12.2	80.0
124	79.0	41.0	male	77.8	6	16.3	95.0
125	76.0	40.0	male	77.8	6	8.0	0.0
105	74.0	32.0	male	71.3	2	7.5	0.0
135	74.0	37.0	male	74.4	8	9.7	58.0

TABLE VII

ANTERIOR PITUITARY INDUCED OVULATION IN FEBRUARY

No.	Body Length (Mm.)	Body Weight (Gm.)	Source of Pituitary		Weight of Pituitary (Mg.)	Total Ovarian Weight (Gm.)	Percentage of Ovulation
			Sex	Length (Mm.)			
D	91.0	43.50	female	71.66	1	18.5	100.0
D1	88.0	50.0	female	86.79	1	21.0	100.0
D2	93.0	62.5	female	71.66	2	23.5	91.3
D3	86.0	39.5	female	82.50	2	13.5	100.0
D4	96.0	62.5	female	77.00	3	21.3	100.0
D5	90.0	45.0	female	79.75	4	19.5	100.0
Ax	91.0	53.5	female	84.65	6	15.0	100.0
Bx	88.0	47.0	female	86.79	18	15.5	100.0
Cx	95.0	59.0	female	86.79	10	24.0	96.0
D10	88.0	64.0	male	81.05	1	16.0	100.0
D11	86.0	53.0	male	71.66	1	13.5	52.6
D6	89.0	43.5	male	71.40	2	10.5	76.2
D7	89.0	47.0	male	81.05	2	15.5	100.0
D8	86.0	49.0	male	75.55	3	12.0	39.1
C9	87.0	43.0	male	81.05	4	16.2	100.0
D9	91.0	49.5	male	75.55	4	21.5	100.0
A9	84.0	46.0	female	76.47	1	18.0	87.5
C	83.0	36.5	female	71.66	1	14.2	100.0
C2	84.0	39.0	female	71.66	2	11.0	100.0
C3	80.0	35.5	female	82.50	2	12.3	95.0
C4	80.0	33.5	female	77.00	3	10.2	100.0
C11	80.0	38.5	male	81.05	1	8.75	39.2
C6	80.0	35.5	male	71.40	2	8.70	100.0
C7	80.0	30.0	male	81.05	2	13.5	100.0
C8	80.0	38.0	male	75.55	3	10.6	100.0
B1	79.0	32.0	female	86.79	1	7.0	100.0
C1	78.0	29.5	female	86.79	1	10.7	100.0
B3	77.0	36.0	female	82.50	2	8.2	100.0
B4	78.0	36.0	female	77.00	3	10.0	100.0
B5	78.0	32.0	female	82.50	4	8.0	100.0
C5	79.0	34.0	female	82.50	4	5.0	100.0
B10	78.0	33.0	male	71.40	1	5.5	100.0
B11	76.0	29.5	male	81.05	1	5.5	0.0
C10	79.0	41.0	male	71.40	1	6.0	100.0
B6	76.0	27.5	male	71.40	2	10.1	100.0
A7	77.0	32.0	male	81.05	2	4.75*	0.0
B7	78.0	34.0	male	81.05	2	10.5	100.0
B8	76.0	27.5	male	75.55	3	8.0	100.0
A	72.0	25.0	female	71.66	1	6.6	92.0
A1	73.0	25.0	female	86.79	1	8.7	94.0
A2	72.0	29.0	female	77.00	2	5.5	100.0
B2	73.0	29.0	female	71.66	2	9.3	54.5
A3	71.0	24.0	female	82.50	2	6.6	96.0
A4	67.0	24.0	female	77.00	3	7.5	100.0
A5	67.0	18.0	female	82.50	4	5.7	100.0
A10	71.0	25.0	male	71.40	1	2.5*	0.0
A11	71.0	23.0	male	81.05	1	5.75	100.0
A6	71.0	23.0	male	71.40	2	5.8	56.6
B9	74.0	26.0	male	81.05	2	5.0	25.0
A8	70.0	26.0	male	75.55	3	5.1	0.0

* Ovaries decidedly undeveloped.

TABLE VIII

POTENCY OF ANTERIOR PITUITARIES REMOVED FROM FEMALES AFTER OVULATION

Expt.	Sex	Body Length (Mm.)	Body Weight (Gm.)	Size Donor (Mm.)	Dosage of Pituitary (Mg.)	Ovarian Weight (Gm.)	Percentage of Ovulation
A15	*a*) female	85.0	62.0	71.15	1	19.5	89.4
	b) female	85.0	57.0	71.15	1	20.0	2.63
	c) female	85.0	61.0	Ringer's	1 cc.	18.5 (control)	0.0
	d) male	Received 1 mg. pituitary; amplexus with (*a*) female in 20 hours					
A16	*a*) female	90.0	76.0	71.15	2	28.5	57.1
	b) female	92.0	73.0	Ringer's	2 cc.	29.0 (control)	0.0
B15	*a*) female	89.0	74.0	76.15	1	22.0	100.0
	b) female	87.0	64.0	76.15	1	18.0	100.0
	c) female	89.0	76.0	Ringer's	1 cc.	18.5 (control)	0.0
	d) male	Received 1 mg. pituitary; amplexus in 23 hours with (*b*)					
B16	*a*) female	88.0	79.0	76.15	2	18.0	100.0
	b) female	88.0	70.0	76.15	2	18.5	100.0
	c) female	89.0	81.0	Ringer's	2 cc.	20.2 (control)	0.0
C15	*a*) female	84.0	57.0	81.22	1	15.0	100.0
	b) female	87.0	73.0	81.22	1	19.0	100.0
	c) female	85.0	68.0	Ringer's	1 cc.	18.5 (control)	0.0
	d) male	Received 1 mg. pituitary; amplexus in 24 hours with (*b*)					
C16	*a*) female	88.0	67.0	81.22	2	17.0	100.0
	b) female	88.0	68.0	81.22	2	18.0	100.0
	c) female	89.0	71.0	Ringer's	2 cc.	19.3 (control)	0.0
D15	*a*) female	90.0	62.0	89.62	1	21.2	100.0
	b) female	87.0	43.0	89.62	1	11.5	100.0
	c) female	88.0	57.0	Ringer's	1 cc.	18.3 (control)	0.0
	d) male	Received 1 mg. pituitary; amplexus within 24 hours with (*a*)					
D16	*a*) female	85.0	55.0	89.62	2	16.0	100.0
	b) female	87.0	65.0	89.62	2	19.0	100.0
	c) female	87.0	57.0	Ringer's	2 cc.	18.8 (control)	0.0
	d) male	Received 2 mg. pituitary; amplexus with (*a*) within 24 hours					

DISCUSSION

BODY-LENGTH AND PITUITARY-WEIGHT RELATIONS

It can be assumed that the body length of the mature frog, as measured from snout to cloaca, will remain relatively constant throughout the year. Any skeletal growth which would alter these dimensions could hardly occur during hibernation, the period of these observations. If, then, we consider body length (as defined) as the constant by which we gauge the other variables—body weight, gonad weight, and anterior pituitary weight—we find that the data accumulated can be resolved into graphs which reveal clearly certain quantitative (and qualitative) variations previously suspected.

The established differences in potency of the male and the female anterior pituitary glands, in respect to inducing sexual reactions, direct us to the quantitative determina-

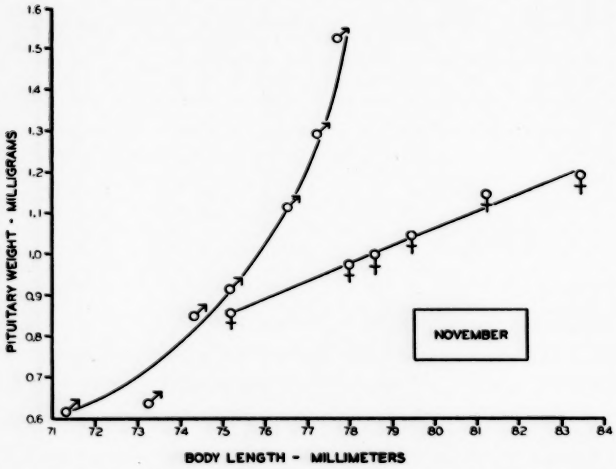

FIG. 1.—A correlation of body length with anterior pituitary weight of males and females in November.

tions on the size of the gland as taken from the two sexes. At the beginning of hibernation (November) the average body lengths of male frogs ranged from 71.3 to 77.8 mm., with a general average of 75.10 mm.; while similar measurements on females showed a range of from 75.2 to 83.5 mm., with an average of 77.67 mm. If the data are arranged as in Figure 1, it can readily be seen that only males and females of body length 74.5 mm. are comparable as to anterior pituitary weights. Subsequent discussion will point out the fact that frogs of body lengths much less than this do not have pituitaries with any appreciable amount of the sex-stimulating hormone. It is possible, then, that frogs measuring up to 71 mm. can be considered immature and that their anterior pituitaries elaborate only the growth hormone which might affect the gonads. This suggestion is substantiated by data that are to be presented in another paper on changes associated with the metamorphosis of the bullfrog, *R. catesbiana*. Between body lengths of 71–74 mm., the sex-stimulating hormone first appears; but up to 74.5-mm. body length there

is little quantitative difference between the amount of gland tissue in males and females. However, Figure 1 indicates that the male anterior pituitary weights lie on a curve reaching a maximum of 1.52 mg. for the pituitary weight of frogs of 77.8 mm. in body length, while the female points lie on practically a straight line reaching a maximum of 1.175 mg. for pituitary weight of frogs measuring 81.05 mm. in body length.

It is clear that, in so far as anterior pituitary weights can be considered equivalent in November males and females, we are limited to frogs of 74.5-mm. body length or less. The average male, however, was 75.10 mm. long and had an anterior pituitary weighing 0.945 mg., while the average female was 77.67 mm. long and had a pituitary weighing 0.960 mg. It would appear, then, that average frogs in November had body lengths and pituitary weights favoring the females. However, the gland weights of larger-than-average frogs greatly favored the males. Males the size of the average female (77.67 mm.) had anterior pituitaries 37 per cent heavier than the females. The largest males were

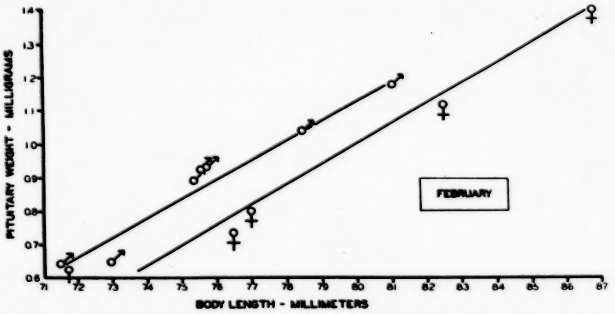

FIG. 2.—A correlation of body length with anterior pituitary weight of males and females in February

5.7 mm. shorter than the largest females, but their anterior pituitaries were 28 per cent heavier than those of the largest females.

The ratio of body length to anterior pituitary weight in February is indicated in Figure 2. The smallest males (71.40 mm.) and females (71.66 mm.) had anterior pituitaries of practically the same weight (0.645 and 0.626 mg.). Again it is suggested that frogs of this size, and shorter, may elaborate from their pituitaries only the growth hormone, and that there exists no essential quantitative or qualitative difference in the glands as studied from either sex.

The average male in February was 75.77 mm. long and possessed an anterior pituitary weighing 0.892 mg., while the average female was 78.88 mm. long and had a pituitary weighing 0.944 mg. The females, then, were 3.11 mm. longer (5.0 per cent), and their anterior pituitaries were 0.052 mg. heavier (5.9 per cent). However, if equivalent body lengths are compared, we note that between the shortest and the longest of the frogs, the male anterior pituitary was consistently heavier than that of the female, the range being from 0.13 to 0.14 mg. Below the sex-maturity level (74 mm.) these differences are much reduced.

It must be pointed out that the longest males were never as long as the longest females. The male maximum was 81.05 mm., while the body-length measurement of the

longest females was 86.79 mm. If we refer only to the longest frogs, we find that the anterior pituitary of the females was 19 per cent heavier than that of the males.

If we compare males alone in November and February (as in Fig. 3), we find that the average body length of the two sets of frogs differed by only 0.67 mm. (75.10 mm. in November and 75.77 mm. in February) and that their anterior pituitaries showed a decrease over the hibernation period of only 0.025 mg. There is, however, a slight decrease in anterior pituitary weight in frogs of equivalent body length, except in frogs shorter than 74 mm. Again it is suggested that the glands of shorter frogs are more comparable, and probably elaborate the growth more, than the sex hormone. The largest males in November (77.80 mm.) had anterior pituitaries 29 per cent heavier than the largest males in February (81.05 mm.), even though they were 3.25 mm. shorter.

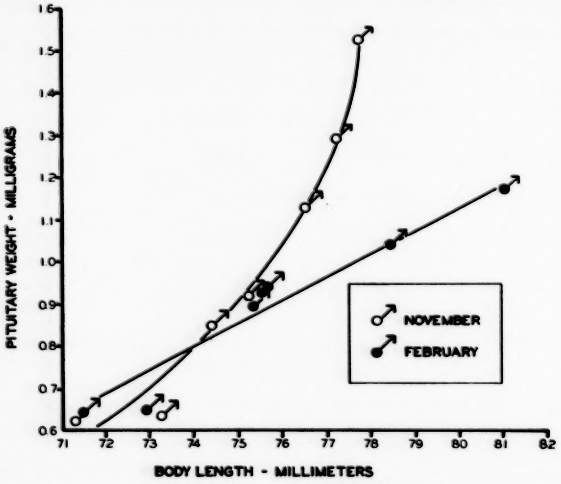

FIG. 3.—A comparison of body-length and pituitary-weight relations of male frogs in November and February.

In comparing the females in November and February (Fig. 4), we find a similar reduction in the relative weight of the anterior pituitary over the hibernation period. The average body length showed an increase of 1.21 mm. and a decrease of 0.018 mg. in anterior pituitary weight. If frogs of equivalent body length are considered, we find the greatest differences in pituitary weight among the smaller mature females. The November females had glands 0.1 mg. heavier at 75.3-mm. body length, and the glands were practically the same weight at 83.5-mm. body length in both November and February. While the largest females seemed to be comparable in the two periods, the same could not be said for the male frogs. In both cases there is indicated an appreciable decrease in average pituitary weight at specific body lengths during the hibernation period.

BODY-LENGTH AND GONAD-WEIGHT RELATIONS

The testes weights were not considered because there was no correlation between body length and gonads of varying sizes, all providing functional sperm. Size or weight,

therefore, could not be considered indications of maturity. However, in relation to the ovaries, a study of Tables II and III will show some correlation between body length and ovarian weight. These ovarian weights were carefully determined, each ovary being weighed to one-tenth of a gram. If the data is arranged as in Figure 5, it will be noted that there is a decrease in relative weight of ovarian tissue between November and February. The February females averaged 1.21 mm. longer, in body size, than those in

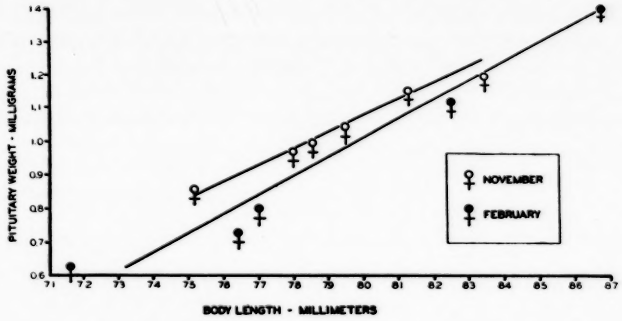

FIG. 4.—A comparison of body-length and pituitary-weight relations of female frogs in November and February.

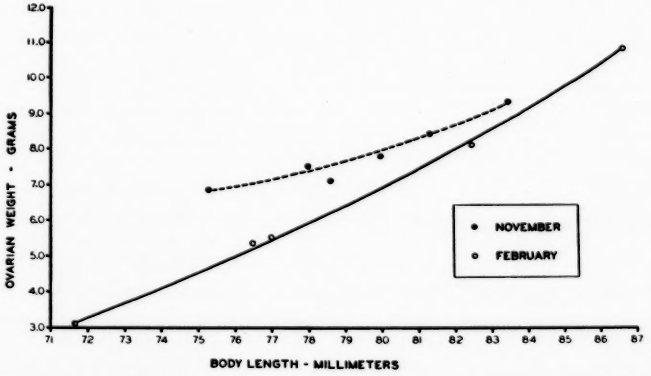

FIG. 5.—A comparison of body length and ovarian weight in November and February

November; but their total body weight was 8.6 per cent less and their average ovarian weight was reduced by 16.1 per cent. During the hibernation period, frogs of 75-mm. body length lost 32 per cent of their ovarian weight, and those of 84-mm. body length lost only 7 per cent. It is to be expected that the soft tissues of the body should lose weight during hibernation, but it could not have been predicted that the ovarian loss would be between 7 and 32 per cent of its original weight, as compared with the 8.6 per cent loss in total body weight, including the ovaries.

Subsequent data will indicate that frogs with ovaries less than 4 gm. in weight are sexually immature. According to Figure 5, such frogs would measure about 74 mm. in body length. It was suggested above, in connection with the anterior pituitary relations, that between 71- and 74-mm. body length the frogs would become sexually mature. The hibernation reduction in weight of both the anterior pituitary and the ovaries is relatively greatest among the smaller frogs.

OVARIAN-WEIGHT AND ANTERIOR-PITUITARY-WEIGHT RELATIONS

Since there were reductions in relative weights of both the ovaries and the anterior pituitaries over the hibernation period, an attempt to correlate these two variables is made in Figure 6. A straight line can be drawn to include all of the points from both November and February. This may mean a functional correlation between the anterior pituitary and the ovaries, but more likely a correlating reduction in relative weight through dehydration or inanition. The reduction of anterior pituitary weight may mean a concentration of glandular material. This is somewhat substantiated by ovulation experiments which are to be reported later in this paper. It can hardly be assumed that ovarian material becomes similarly concentrated over the hibernation period. A correlated study of the ovaries from the physiological, histological, and even cytological points of view, covering the entire year, is certainly in order.

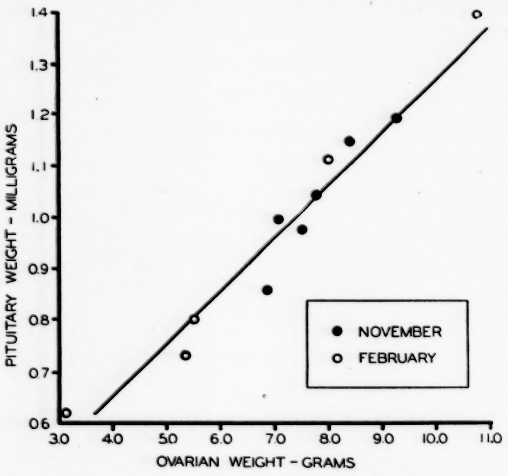

FIG. 6.—A comparison of ovarian weight and pituitary weight in November and February.

OVULATION AS INDUCED BY ANTERIOR PITUITARY INJECTION

The major purpose of these experiments was to determine the relative potencies per unit of weight of the male and female anterior pituitaries in respect to inducing ovulation. Females were segregated into body-length groups similar to the groupings of donors listed above. The measurements on the living frogs were checked with post-mortem examinations. Frogs were used within 24 hours after receipt at the laboratory, since it is well known that frogs kept at laboratory temperatures rapidly lose their susceptibility to ovulation induction. With *R. pipiens* the eggs are normally liberated immediately after the hibernation period, before the frog has consumed all of its stored food. If, then, ovulation is delayed and the frogs do not acquire an appropriate amount of fresh food, it is natural that the yolk stored within the eggs would be consumed by the host. Ovaries of females kept in the laboratory for a week or two show degeneration changes.

The percentage of ovulation was determined by allowing adequate time for complete reaction to the injected hormone and then weighing the eggs in the body cavity and uteri.

The weight of the original, pre-ovulation ovaries was then estimated by adding the weight of the post-ovulation ovaries to the weight of the ovulated eggs. Ovarian tissue was estimated at 0.5 gm. in each case and was not considered in the calculations of ovulation percentages.

Ovulation induced in November.—Laboratory experience indicated that the injection of a single gland from either male or female in November was inadequate to induce any ovulation; hence dosages were ranged from 2 mg. of gland tissue up to 8 mg. It must be remembered that male glands range in weight from 0.615 to 1.520 mg. and that female glands range from 0.860 to 1.190 mg. each (see Tables II and III). In Table VI the data are so arranged that the equivalent-sized recipients are grouped together in sequence of increasing dosages of anterior pituitary. Those receiving the hormone from males are separated from those receiving the hormone from females. In almost all instances an increase in ovulation percentage can be noted with increasing dosages, regardless of the

TABLE IX

INDUCED OVULATION IN NOVEMBER

Size Range of Recipients (Mm.)	No. of Females Used	Donor of Pituitary	Dosage of Pituitary (Mg.)	Percentage of Ovulation
74–94	5	males	2	0.0
78–93	5	males	4	7.7
79–94	5	males	5	27.0
76–92	5	males	6	36.6
74–91	5	males	8	41.4
77–92	5	females	2	0.0
83–97	4	females	3	23.7
77–98	5	females	4	34.2
77–92	5	females	5	88.0

source of the pituitary. This has previously been suggested (Rugh, 1934), the ovulation reaction being quite different from an all-or-none reaction. It is readily apparent, from data in Table VI, that response to the male gland, while not qualitatively different from response to the female gland, is certainly quantitatively different when equivalent doses are considered. In no instance was 2 mg. of the male gland adequate to induce any ovulation; and in only two instances did 4 mg. of the male gland elicite any reaction, the maximum being 8 per cent ovulation. While 2 mg. of the female gland was never adequate in November to induce any ovulation, 3 and 4 mg. were sufficient to elicite a reaction in most cases. In some instances these doses were adequate to induce as much as 95 per cent ovulation (see frogs Nos. 9 and 14). Between 6 and 8 mg. of male gland was necessary to induce as much as 80 per cent ovulation (and this in only a few cases), while 5 mg. of the female gland generally caused more than 90 per cent ovulation. There is no doubt but that there is a potency difference between the male and the female glands in respect to inducing ovulation.

In Table IX there is summarized the data of Table VI in such a manner as to emphasize the dosage of anterior pituitary.

There is another aspect of the anterior-pituitary–ovulation reaction which, if we arrange the same data (Table VI) in such a manner as to segregate females of similar ovari-

an weight, indicates clearly that the size of the ovary (weight) is a very important factor in regard to its susceptibility to anterior-pituitary-induced ovulation. If we arbitrarily select the ovarian weight ranges of 6.0–9.9 gm., 10.0–14.9 gm., 15.0–19.9 gm., and 20.0+ gm., the gradual increase of susceptibility with increase of ovarian weight is quite apparent (Table X).

One would expect some correlation between body length and ovarian weight, and in general it can be said that the larger females tend to have larger ovaries. However, there is no rigid correlation between these two factors, for female No. 111 (Table VI) was 93 mm. long and had ovaries weighing 10.1 gm. while female No. 124 was 79 mm. long and had ovaries weighing 16.3 gm. Females Nos. 13 and 14 had the same body length, had ovaries within 2.5 gm. of being the same weight (well over the minimal level of maturity),

TABLE X

OVARIAN WEIGHT IN RELATION TO INDUCED OVULATION (NOVEMBER)

No. of Cases	Ovarian Weight (Average) (Gm.)	Range of Ovarian Weight (Gm.)	Sex of Donor	Dosage of Pituitary (Average) (Mg.)	Percentage of Ovulation
6	7.78	6.0–9.9	male	4.83	9.66
12	11.92	9.9–14.9	male	4.91	14.96
4	16.22	14.9–19.9	male	4.50	24.20
3	22.7	20.0+	male	6.33	76.60
1	8.00	6.0–9.9	female	2.00	0.0
8	11.88	9.9–14.9	female	3.15	11.12
9	17.97	14.9–19.9	female	3.88	58.54
1	24.50	20.0+	female	5.00	95.00

and each received 4 mg. of female anterior pituitary, and yet there was a difference of 95 per cent in the ovulation reaction. The comparative reactions of frogs Nos. 117 and 118, Nos. 8 and 9, Nos. 124 and 125 and Nos. 132 and 134 (Table VI) seem to indicate ovulation differences which might be attributed solely to differences in ovarian weight. In many instances, however, ovarian weight will give no clue as to the expected degree of ovulation.

There is still another possible explanation of differences in reaction by similar frogs to similar doses of anterior pituitary. If frogs Nos. 8 and 9 and Nos. 13 and 14 (Table VI) are compared, it will be noted that in each case the female that gave the greatest ovulation had received the hormone from the larger donors. It is, of course, possible that larger frogs may have more of the sex-stimulating factor per unit of weight of the anterior pituitaries than do the smaller, but mature, frogs. There is considerably more evidence of this in the February ovulations, but the foregoing pairs suggest it even in November.

If we disregard the ovarian weight of the recipient and the body size of the donor of the anterior pituitary hormone, we can plot percentage of ovulation against milligrams of injected gland tissue, as shown in Figure 7. The male and female points tend to converge near the 2–3-mg. level, but the inclination of the female points is very much greater than that of the male points.

Ovulation induced in February.—It was anticipated that ovulation could be induced in February with smaller doses of anterior pituitary from either male or female donors;

hence the dose was started at 1 mg. Among the February donors the males varied in length from 66.00 to 87.00 mm. and had anterior pituitaries ranging from 0.645 to 1.175 mg. in weight. This scale of pituitary weights is relatively somewhat lower, in respect to body length, than the comparative weights for November, as is indicated in Figure 3. However, the two lines converge near body length 74 mm., as has been pointed out under the discussion of pituitary weights and maturity. The females in February varied in body length from 68.00 to 92.00 mm., and their pituitaries from 0.6266 to 1.395 mg. Here again the February points fall on a line considerably below that for November (see Fig. 4). In Table VII it will be noted that in only 4 females out of 50 there was no ovulation at all, and in all 4 cases the ovaries weighed less than 5.5 gm. The level of maturity

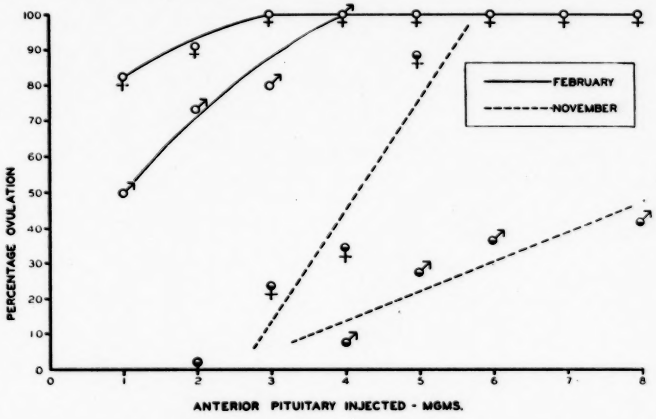

FIG. 7.—Ovulation as induced by varying doses of male and female anterior pituitaries in November and February.

is in the vicinity of 5 gm. Since in 32 of the 50 cases there was 100 per cent ovulation, we must certainly assume very great differences in the reaction in November and in February. Those cases which did not give complete ovulation became, therefore, the critically important cases.

If the data are arranged so that we can compare increasing doses of anterior pituitary with ovulation percentage, regardless of other factors, we see again (as in Table IX for November) that with increasing doses there is increasing ovulation. These data are summarized in Table XI.

Reference is again made to Figure 7, on which these points are plotted. Since one of the females receiving 3 mg. of male pituitary had ovaries weighing 5.1 gm. and which appeared to be very immature, this one case was omitted from the graph. Four milligrams of anterior pituitary from either males or females was an adequate dose to induce 100 per cent ovulation in February, while the same dose from males produced only 7.7 per cent ovulation and from females produced 34.2 per cent ovulation in November. It must be pointed out here, however, that a number of factors may be involved. First, there may be a concentration of the anterior pituitary hormone through the loss in total weight of the gland over the hibernation period. Second, the ovaries may have become more sus-

ceptible to equivalent doses of the hormone. Third, the host pituitary may have become active in anticipation of normal breeding in the spring.

If we arrange the data of Table VII in such a manner as to group together females with approximately the same ovarian weights, we note that in general the larger ovaries are the more susceptible to anterior pituitary stimulation. This was shown for Novem-

TABLE XI

INDUCED OVULATION IN FEBRUARY

Size of Recipient (Mm.)	No. of Females Used	Donor of Pituitary	Dosage of Pituitary (Mg.)	Percentage of Ovulation
71–88	8	males	1	61.4
71–89	7	males	2	73.1
70–86	4	males	3	59.8 (*79.0)
67–91	3	males	4	100.0
72–91	8	females	1	96.6
71–93	8	females	2	92.0
67–96	4	females	3	100.0
67–90	4	females	4	100.0
91	1	females	6	100.0
88	1	females	8	100.0
95	1	females	10	96.0

*One female with decidedly immature ovaries. If this female is not included, the ovulation is 79 per cent.

TABLE XII

OVARIAN WEIGHT IN RELATION TO INDUCED OVULATION (FEBRUARY)

No. of Cases	Ovarian Weight (Average) (Gm.)	Range of Ovarian Weight (Gm.)	Sex of Donor	Dosage of Pituitary (Average) (Mg.)	Percentage of Ovulation
12	5.94	4.75–9.9	male	1.65	51.7
7	11.53	10.00–14.9	male	2.14	81.1
3	15.90	15.00–19.9	male	2.33	100.0
1	21.50	20.00+	male	4.00	100.0
11	7.1	5.00–9.9	female	2.36	100.0
7	11.7	10.00–14.9	female	2.00	99.3
5	17.3	15.00–19.9	female	4.00	97.6
4	22.45	20.00+	female	4.00	99.3

ber in Table X. Table XII gives these data. Here, as in the November data, we find suggestions that the source of the anterior pituitary may be responsible for certain differences in reaction. Compare, for instance, females D2 and D3, D10 and D11, D6 and D7, and B2 and A3 (Table VII). However, there are other instances in which this does not seem to be the major factor, as in A6 and B9. In this case it appears that, while there was greater ovulation in A6, the ovaries of the other frog are almost too light to be considered mature.

THE POTENCY OF POST-OVULATION PITUITARIES

It has previously been suggested that induced ovulation may have no effect on the host's anterior pituitary, that the injection of the hormone is similar to the liberation of the hormone by the host. Cytological evidence for this is being accumulated; but since 43 ovulating females were available through the previous experiments, their pituitaries were removed and weighed in exactly the same manner as for the original donors. The data in Table VIII show clearly that the anterior pituitaries of females which had been induced to ovulate did not lose in potency. The control females were injected with an equivalent volume of Holtfreter's modification of amphibian Ringer's, and in no case did they show any ovulation. Adult males were also injected, and in all cases amplexus was induced. It will be noted that in Experiment A15 the male was in amplexus with the most actively ovulating female. This single case may not be significant, but it has been our general experience that amplexus can be achieved only with sexually stimulated females of *R. pipiens*.

SUMMARY AND CONCLUSIONS

1. Up to 70-mm. body length, frogs may be considered sexually immature; and between 70 and 74 mm., the body length and anterior pituitary weight relations of males and females are comparable. The glands show correlating differences in sex-stimulating hormone.

2. In November the largest males were 5.7 mm. shorter, and their anterior pituitaries were 28 per cent heavier, than those of the largest females. In February the average female was longer and had a heavier anterior pituitary than the average male, but frogs of equivalent body length showed anterior pituitary weights favoring the males by 0.13–0.14 mg. The largest frogs are always females.

3. Both males and females show decrease in relative weight of their anterior pituitaries, as compared with body length, during hibernation. The reduction is as much as 29 per cent in males.

4. Ovarian-weight reduction ranged from 7 to 32 per cent during hibernation, accompanied by an average reduction of only 8.6 per cent in total body weight. Pituitary and ovarian weight changes are correlated.

5. Five milligrams of male anterior pituitary is equivalent to about 3 mg. of female gland tissue in respect to inducing ovulation in November. The average male gland is 16 per cent heavier and 60 per cent as potent as the female gland, per unit of weight. Increasing doses of anterior pituitary from either males or females will induce increasing percentages of ovulation in either November or February.

6. The larger ovaries are the more susceptible to equivalent doses of anterior pituitary in both November and February. Ovaries less than 5.0 gm. are generally immature. The heaviest ovaries were 24.0 gm. There is no rigid correlation between body length and ovarian weight, although the largest females tend to have the larger ovaries.

7. Four milligrams of male gland will induce 7.7 per cent ovulation in November, while an equivalent weight of female gland will induce 34 per cent ovulation. In February these doses, from either males or females, will induce 100 per cent ovulation.

8. The potency of anterior pituitaries of females after they have been induced to ovulate seems not to be reduced in the least. The injection of hormone may be comparable to the liberation of the host's hormone in normal breeding, and the host's gland is unaffected.

9. The variables considered in these observations, all of which have a bearing on the pituitary-ovulation reaction, are:

A. Anterior pituitary hormone:
   1. Size and sexual maturity of donor
   2. Concentration of hormone through hibernation reduction in anterior pituitary weight
   3. Activity of recipient's anterior pituitary
   4. Source of hormone (from male or female)
   5. Dose of hormone (in milligrams)

B. Recipient:
   1. Size (body length) and sexual maturity
   2. Size (weight) and susceptibility of ovaries to ovulation induction

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THE MODIFICATION OF SEX DEVELOPMENT IN THE CHICK EMBRYO BY MALE AND FEMALE SEX HORMONES[1]

(Twenty figures)

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THE interpretation by Lillie (1916, 1917) that the freemartin is the result of the action of male sex hormones led to the belief that the nature of the processes involved in embryonic sex-transformation might be analyzed experimentally in the laboratory. As a consequence, experiments were devised to duplicate in the chick embryo Nature's experiment in cattle twins. By grafting gonad tissue, either embryonic or adult, to a host embryo, a relationship can be set up which simulates rather closely that existing between cattle twins *in utero*, inasmuch as the significant means of communication between graft and host is by way of the blood circulation of the embryonic membranes. Minoura (1921), the first to make such an analysis, found intersexual structures of various grades which he believed were produced by the action of sex hormones from grafts of pieces of testis or ovary of the adult fowl. Such sex-modifications were subsequently shown not to be specific sex-hormone effects since they occur in the normal embryo with an 18 per cent frequency, which may be readily increased by certain conditions of operation and incubation, especially by low temperature at a critical period in development (Willier and Yuh, 1928; see Willier, 1932, pp. 143–49, for a review of this topic). Furthermore, in reciprocal experiments it has been shown that the gonad-forming area or rudiment of indifferent stages fails to undergo sex-reversal if grafted to the chorio-allantois of host embryos already differentiated as to sex (Willier 1927, 1933). Self-differentiation of the indifferent rudiment or area occurs regardless of the sex of the host embryo. In one exceptional case among these, an ovotestis differentiated from a gonad-forming area of a 31-somite donor grown for 9 days on a female host. In this case the gonad area was apparently determined as a left testis, the rudimentary cortical component of which was activated by female sex hormones of the host.

The general lack of cases of sex-modification in the chick embryo has led to various hypotheses. One view is that sex hormones are absent, the early differentiation being dependent entirely upon genetic determiners of sex. Another is to the effect that, if sex hormones are present, they fail to participate in the processes of sex-differentiation in the embryo. Still a third view is that embryonic sex hormones are either different or have a different mode of action from the sex hormones which have been demonstrated to play such an important rôle in controlling sex characters in the chick after hatching. Such conclusions are not justified on the basis of what is known concerning sex-reversal

[1] This investigation was aided in part by the University of Rochester and in part by a grant to the University of Chicago from the Rockefeller Foundation and the National Research Council. In the histological study of the embryos we are greatly indebted to Dr. Mary E. Rawles for assistance of a high grade.

in the amphibian embryo, where reversal of sex may be readily induced by methods of grafting similar to those used in the chick, and by parabiosis. Control of the direction of sex-differentiation by the action of sex hormones has thus been demonstrated for this group of animals.

The object of this investigation is to determine what effects, if any, the injection of male and female hormone solutions into the hen's egg at an early stage of development (i.e., prior to the beginning of sex-differentiation) would have upon the developing sex glands and ducts. It is readily seen that such an analysis would be of considerable theoretic interest in itself, since these hormones are known to play an important rôle in controlling secondary sex characters in the chick after hatching. In addition, the possibility that these substances might modify the direction of differentiation of the gonad rudiment, such as occurs in the freemartin, seemed worth testing. Furthermore, it was hoped that from such an investigation some light would be thrown upon (1) the singular lack of sex-modification in the chick embryo when the grafting method is used, and (2) upon the nature of the mechanism of the sex-differentiating process.

The initial experiments of this analysis were carried out by Kozelka and Gallagher (1934), with several significant results. First, theelin, theelol, and an extract of male human urine (containing some estrogenic substances) brought about, in a number of cases, embryos in which the left gonad is an ovotestis and the right one entirely testicular. Second, male-urine extracts produced an "abnormality of Müllerian ducts" in the males and in some cases an increase in diameter of the Wolffian ducts. Third, bull-testis extracts had no effect upon the sex apparatus except that "a slight effect was manifest on the embryonic ducts" (p. 1144).

These results seemed significant enough to warrant a study of this problem on a broader scale and scope. Consequently, various concentrations of crystalline theelin, crystalline theelol, bull-testis extracts, and a variety of male human urine preparations were tested. The solvents of the hormones used were also injected into control embryos to ascertain whether they possess any specific action on the developing genital system. In addition a careful study was made of the sex glands and ducts of normal control embryos. Lastly, in order to make sure of the original sex-determination of the embryo, the precaution was taken in one set of experiments to use eggs from a cross, the embryos of which are distinguishable as to sex by a sex-linked plumage character. The principal generalizations of these studies were presented in April, 1935, before the Society of Biochemists and the American Association of Anatomists, and later communicated to the National Academy of Sciences (Willier, Gallagher, and Koch, 1935*a*, 1935*b*, 1935*c*).

METHODS OF INJECTION AND MATERIAL[2]

The hormone solutions were injected into the albumen of eggs which had been incubated for approximately 48 hours. This stage was selected since it is the earliest at which sterility can be detected by candling over a strong light, thereby conserving the supply of hormones. The pointed end of the egg was cleaned by wiping with cotton moistened with ethyl alcohol, and the shell was punctured by an automatic blood lancet. One-tenth cubic centimeter of the solution to be tested was then introduced by means of a syringe fitted with a No. 24 gauge needle which had been shortened so that the tip was

[2] The injections and a preliminary examination of the embryos were made during May, 1934, at the University of Illinois. We are deeply indebted to Dr. Robert Graham, of the department of animal pathology, for granting us the use of an accurately controlled egg incubator and other facilities.

only 0.5 cm. from the hilt. The use of the short needle prevented penetration into the yolk and possible injury to the blastoderm. Only rarely during the injection did any fluid exude from the shell opening. The small hole in the shell was closed immediately after injection by means of melted paraffin.

The egg was returned to the incubator and incubated until the nineteenth or twentieth day. At this time the number of living embryos was ascertained and each embryo was dissected and examined under a binocular dissecting microscope for any macroscopic changes in the reproductive glands, ducts, or any of the other organs. After recording the findings, the embryos, *in toto*, were fixed in Bouin's solution (in a few significant cases the gonads were fixed separately). In a large number of cases the gonads and portions of the Wolffian and Müllerian ducts were histologically examined.

The great majority of eggs used were of the single-comb Brown Leghorn breed. In one set of experiments the eggs came from crosses made between two breeds of fowl in such a way that the sex of the resulting embryos could be distinguished by sex-linked plumage characters. For example, Rhode Island Red males crossed with Barred Rock females give female embryos with entirely black plumage and males black all over except for a patch of white plumage on top of the head. In another cross (White Wyandotte males × Rhode Island Red females) the plumage character was found, in both the control and experimental embryos, to be less reliable and not useful as an indicator of sex, presumably because the parents were not of pure stock.

EFFECTS OF THEELIN AND THEELOL

A total of 173 embryos has been examined macroscopically for modifications in the urinogenital system brought about by the injection of theelin and theelol. Two classes of embryos are revealed: those in which the gonads and ducts of both sexes are normal and those in which an intersexual condition of the sex glands and ducts is exhibited. Of these, 81 are interpreted as zygotic males and 92 as zygotic females, giving a sex ratio of 46.82, the percentage of males. This is a little lower than the normal sex ratio for the chick embryo (see Willier and Yuh, 1928, p. 109). This interpretation of the zygotic nature of sex is based, in 35 embryos (theelin, 7♂:8♀; theelol, 11♂:9♀), upon the presence of sex-linked plumage as well as upon the structure of the sex glands and ducts (8♀ and 10♂ were histologically studied); in 30 embryos (theelin, 10♂:4♀; theelol, 9♂:7♀), upon the histology of the gonad alone. After it became evident that the male gonad undergoes modification while the female gonad does not, a larger number of male gonads was selected for study to ascertain any variation in the histological picture produced by the hormones. In the remainder (theelin, 25♂:40♀; theelol, 19♂:24♀), which were studied only macroscopically, sex is ascertained by the form of the gonads and the condition of the oviducts. The males undergoing sex-reversal may be distinguished from the females as follows: (1) The left gonad, although more or less flattened like a left ovary, is nevertheless thicker and more rounded than normal, owing to the retention of testicular tissue at the hilus (i.e., the testicular form is still retained to a certain degree). (2) The added ovarian cortex is usually less regular in its distribution and may be distinguished in the living embryo as a transparent layer over the more opaque and yellowish testicular tissue. (3) The right testis retains its male form much longer than the left testis; also, its size is almost always greater than a typical right ovary. (4) The oviducts persist and exhibit a higher frequency of abnormalities than oviducts of female embryos.

The experiments are summarized in Table I to show dosage of crystalline female hormone given, solvent used, the number of embryos injected, and the percentage of survival. No essential difference was found in the effects of theelin and theelol; hence the embryos will be grouped together for descriptive purposes.

Zygotically determined males.—In the male embryos the right testis, although generally smalier than normal, is usually normal in shape and histology; whereas the left testis is very large, has the flattened form of a normal left ovary, and is histologically an ovotestis. The striking difference in size and form of the right and left gonads resembles closely similar differences characteristic of the normal female. The persistence of one or both oviducts makes this resemblance still more female-like in character. In fact, upon casual gross examination one is impressed by an apparent change in the sex ratio, the

TABLE I

SUMMARY OF DATA ON EFFECTS OF FEMALE SEX HORMONES

Hormone	Serial No.	Amount Injected (Mg.)†	No. of Eggs Injected	Survival on 19th–20th Day	Percentage of Survival
Theelol	E 16	0.05	40	24	60
	E 15	0.2	40	34	85
	E 27*	1.0	12	9	75
	E 26	2.0	15	11	73
Theelin	E 14	0.05	39	37	95
	E 13	0.2	40	31	77
	E 17	0.4	13	11	65
	E 24*	2.0	17	10	60
	E 25*	1.0	11	6	54
Experimental control‡	E 11 glycol	0.1 cc.	30	29	97

* Sex-linked plumage.

† Dissolved in 0.1 cc. ethylene glycol.

‡ Over 35 other controls have been examined. These include embryos treated with extracts of thymus and of an inactive residue from preparation E 3 and untreated ones (sex-linked) as well.

number of female embryos increasing as the dosage of female hormones is increased, until all embryos might be so classified. Wolffian ducts are apparently not modified in any embryo injected with female sex hormones.

The frequency of modification of the males in the female direction is found to be proportional to the dosage of theelin or theelol given. Some degree of modification of the testis occurred in 6 cases out of 27 injected with 0.05 mg., in 10 out of 15 injected with 0.2 mg., and with dosages ranging from 0.4 to 2.0 mg., every embryo of a group of 23 was affected. These figures are based in half of the cases upon histological, and half upon macroscopic, study.

The best index to the degree of intersexuality attained in the 29 male embryos histologically examined is the composition of the left gonad. For convenience in description the left testes may be grouped in the following grades of transformation: (1) no ovarian cortex; (2) definite ovarian cortex, no medulla; (3) cortex definite, ovarian medulla scanty; (4) cortex and medulla both distinct, testis tissue reduced; (5) cortex and ovarian

medulla predominant, few testicular cords remaining; and (6) cortex and ovarian medulla only (complete reversal).

1. *No ovarian cortex.*—In one embryo receiving a dosage of 0.05 mg. theelin the left testis is normal histologically; and although a definite cortex is lacking, the surface epithelium has persisted in places in its original form, i.e., similar to that found on the testis of a 10-day normal embryo. The right testis is normal histologically. Oviducts are entirely absent, as in normal males.

2. *Ovarian cortex rudimentary; medulla absent.*—In three embryos, each of which received 0.05 mg. theelol, the left gonad is largely composed of testis tissue. However, on the surface are scattered areas of thickened epithelium, which simulates the incipient cortex of the testis primordium of a 10-day embryo. Occasionally beneath such thickenings are found nodules or masses of moderately well-differentiated cortical tissue. The right testis in each case is perfectly normal as to size and structure. Oviducts are absent as in the normal male, with the exception of a cystlike trace situated anteriorly on the left side of one embryo.

3. *Definite cortex; ovarian medulla scanty.*—Four embryos exhibit this grade of transformation (see Fig. 1). Two received injections of 0.2 mg. of theelin; one, 0.4 mg. of theelin; and one, 0.2 mg. of theelol. In each case the left gonad consists primarily of a testis body upon which is superimposed a layer of overian cortex, and between which a very few medullary cords are occasionally found. The cortex consists of ovigerous cords, as in the normal left ovary; but generally they are shorter, less well-developed and less numerous; in some regions of the gonad they are absent altogether. The right testis is structurally normal; in one case it appears to be definitely smaller than normal. Traces of either the right or left or both oviducts are found (2 cases) or they are absent altogether (2 cases).

4. *Ovarian cortex and medulla both distinct; testis tissue reduced.*—Ten embryos belong in this category. The dosages of theelin given were 0.05 mg. (one case), 0.2 mg. (one case), and 0.4 mg. (three cases); of theelol, 0.05 mg. (one case) and 0.2 mg. (four cases). In the left gonad of all these cases (Figs. 2, 3, 15, 16, and 17) the amount of testicular tissue is perceptibly reduced, roughly corresponding to the quantity of ovarian medulla present. The medulla forms a distinct layer, extending in most cases throughout the length of the gonad (Figs. 15, 16, and 17). In the more advanced cases of transformation, the testis tissue is confined largely to the hilus. In fact, the gonads may be arranged in a graded series with respect to the quantity of medulla present. It gradually increases in thickness from a thin layer to a relatively thick one as it spreads toward the hilus. In general the cortex resembles very closely that of a normal left ovary in structure and thickness; in some cases it is a little thinner and the ovigerous cords are fewer and less regularly arranged.

Although in all the embryos under consideration the right gonad (Figs. 2 and 3) has typically the form and structure of a right testis, nevertheless it exhibits certain definite indications of transformation in the female direction. The most striking of these is the development of a few definite medullary cords in two cases, irrespective of whether theelin (0.4 mg.) or theelol (0.2 mg.) was injected. They are found more or less scattered among the testicular cords, especially toward the free surface of the gonad. Such cords are usually somewhat distended, as in a normal ovary. However, some are continuous with testicular cords, suggesting an intermediate stage in their transformation.

A second difference from the normal is that a few testicular cords not only connect

with the free surface of the gonad but appear to open into the coelome. This is found in as many as seven of the ten cases studied. Often, where the cords join with the surface, there is a proliferation of cells which stain darkly. At the surface either on the attached or free sides are also found spaces in the mesenchyme.

A third feature noticed is an increase in the number of germ cells in the testicular cords (Fig. 17). It is definite in three cases (theelol). Finally, it should be noted that no definite ovarian cortex has differentiated on the right testis except for a few lumps on the surface in two embryos (0.2 mg. theelol; 0.4 mg. theelin).

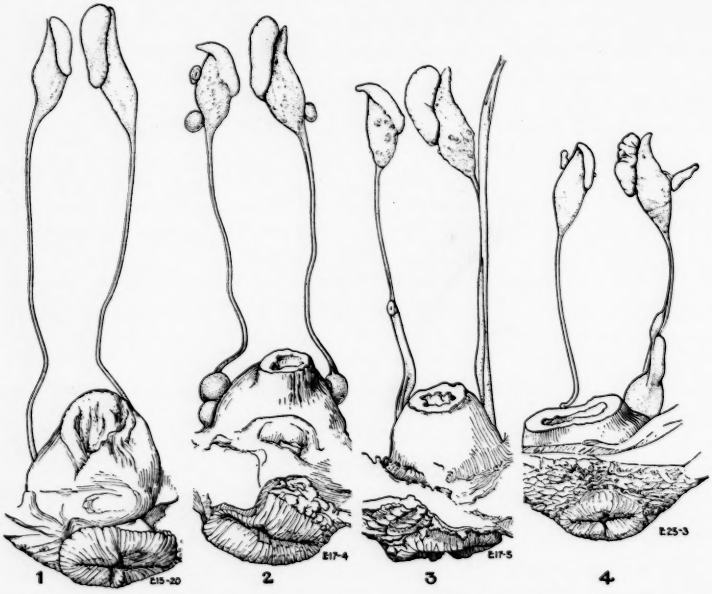


FIG. 1.—Reproductive system of a genetic male embryo (E 15–20) injected with 0.2 mg. theelol. Normal ♂ except for flattening of left gonad (ovotestis). ×3.

FIG. 2.—Reproductive system of a genetic male embryo (E 17–4) injected with 0.4 mg. theelin. Note flattened left gonad (ovotestis) and cystlike portions of oviducts attached lateral to Wolffian bodies and to cloaca. ×3.

FIG. 3.—Reproductive system of a genetic male embryo (E 17–5) treated with 0.4 mg. theelin. Left gonad distinctly flattened, resembling closely a left ovary. Both oviducts persist. ×3.

FIG. 4.—Reproductive system of a genetic male embryo (E 25–3) injected with 1.0 mg. theelin. Left gonad becomes ovary-like; right remains a testis. Portions of both oviducts persist; left swollen. ×2.5.

The oviducts are absent on both sides (four cases), or occur as a rule in the form of short cystlike segments on one or both sides (six embryos—half receiving 0.4 mg. theelin and half 0.2 mg. theelol) (see Fig. 2); in one of these (0.4 mg. theelin) the left oviduct is full length but rudimentary and the right is longer than in normal females (Fig. 3). The segments may be anterior or posterior in position and are often much swollen. It becomes evident from these data that with the higher doses of hormone the oviduct tends to persist.

5. *Ovarian cortex and medulla predominant; few testis cords remain.*—This grade of transformation is found in nine embryos, of which four received theelin and five theelol. The dosages of theelin given were: 0.4 mg. (one case), 1.0 mg. (one case), and 2.0 mg. (two cases); of theelol, 1.0 mg. (two cases) and 2.0 mg. (three cases). The left gonad (Figs. 4 and 5) in all embryos appeared, upon casual examination, to have the structure of a left ovary; but a careful study revealed the presence of a few—in some instances but one or two—typical testicular cords at the hilus. The testicular cords retain their male characters, such as solidity, peripheral arrangement of non-germinal cells, and scattered

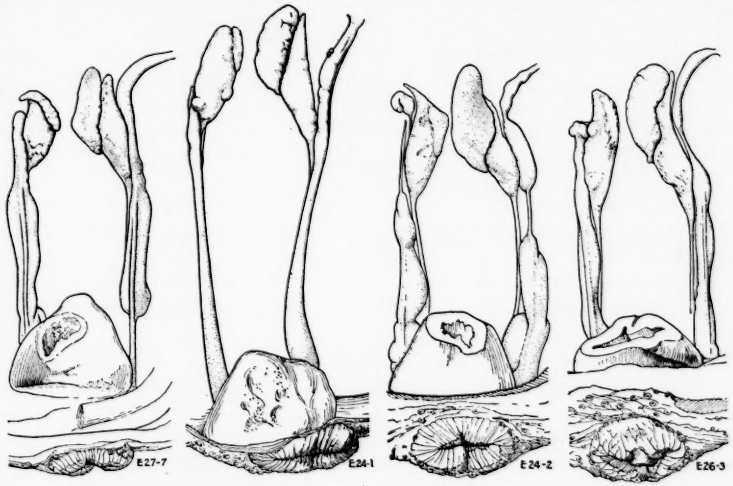


FIG. 5.—Urinogenital system of a genetic male embryo (E 27–7) injected with 1.0 mg. theelol. Both gonads ovary-like; left almost indistinguishable from normal ovary. Both oviducts persist and hypertrophy. ×5.

FIG. 6.—Urinogenital system of a genetic male embryo (E 24–1) injected with 2.0 mg. theelin. Left gonad ovary like, no testicular cords; right likewise becomes ovary-like. Oviducts persist and hypertrophy. ×2.5.

FIG. 7.—Urinogenital system of a genetic female embryo (E 24–2) treated with 2.0 mg. theelin. Normal ♀ except for persistance of right oviduct and hypertrophy of both oviducts. ×2.5.

FIG. 8.—Urinogenital system of a genetic female embryo (E 26–3) injected with 2.0 mg. theelol. Modifications of oviducts similar to preceding figure. ×2.5.

position of germ cells (Fig. 19). The great bulk of the gonad, however, is ovarian medulla and cortex. The medulla usually consists of distended medullary sex cords which are structurally identical with those found in a normal ovary of the same age. In some cases the medulla is made up of solid cords, only a few being distended. In thickness and structure the ovarian cortex resembles that of a normal ovary of a younger embryo. Although some ovigerous cords show groups of germ cells in synaptic stages of meiosis, the majority are in presynaptic stages. See Figures 18 and 19.

In these cases of rather extreme reversal of the left testis, the right testis likewise shows signs of reverting into a right ovary. There are external indications such as reduc-

tion in size and an alteration in shape (Figs. 4 and 5). In five cases it is somewhat smaller than a normal right testis of the same age; occasionally in such cases it is more or less flattened, thus resembling a right ovary. Structurally there are a number of changes, the most striking of which is the appearance of ovarian medulla in the form of distended medullary cords. These are apparently forming from the testicular cords, since sex cords are found which are partly ovarian (hollow) and partly testicular (solid) in form. The number of medullary cords varies from a very few to a condition where they form a prominent part of the gonad structure. As a rule the number of them is quite small. In four cases (two receiving 1.0 mg. of theelin or of theelol; and two, 2.0 mg. of theelin or theelol) none was found. They are more or less scattered, although more likely to occur at the surface than toward the hilus.

Another difference from the normal is the somewhat frequent connection of testicular cords with the surface of the testis. In some cases the incipient lumen appears to connect with the coelome. Finally, in one case a trace of cortex and in two cases small masses of germ cells are found to occur on the ventrolateral surface of the right testis.

The striking feature of this grade of sex-transformation is the invariable persistence and hypertrophy of the oviducts. They persist on both sides of the body in the following forms: (1) as discontinuous short portions distended with liquid, usually situated either anteriorly, lateral to the Wolffian body, or more posteriorly, lateral to the cloaca (Fig. 4); (2) as a short swollen portion on one side and full length on the opposite side but consisting of alternate swollen and non-swollen portions, giving the duct a segmented appearance; (3) as two "segmented" ducts of full length (Fig. 5).

6. *Ovarian cortex and medulla only* (*complete reversal*).—In two embryos (with sex-linked male plumage) which received, respectively, 2.0 mg. of theelin and 1.0 mg. of theelol, the left testis has been completely transformed into an ovary which is similar in all essential respects (structure, shape, and size) to a normal left ovary (Fig. 6). No testicular cords whatsoever are found. The great majority of the germ cells of the cortex are in presynaptic stages of meiosis rather than synaptic stages which are characteristic of the normal ovary of the same age.

The right testis, in both cases, resembles a right ovary in shape and size. It is made up largely of distended medullary cords and a few remaining testicular cords. The latter are found near the hilus. No definite ovarian cortex is found.

The oviducts of these interesting cases exhibit a striking modification. The left is as long as in the normal female but is greatly distended throughout its length (except for the shell gland, which has normal proportions). The right oviduct is also hypertrophied and nearly as long as the left, terminating anteriorly in an ostium like opening (ostial lip much thickened); no shell-gland region is apparent.

Zygotically determined females.—Usually it is readily possible to distinguish the zygotic females from the modified males. Although the left ovotestis resembles the left ovary, the latter is generally more extensive and more flattened. Furthermore, the right gonad of the males is usually testis-like in form, while the right ovary is very flat and thin. It is therefore possible to distinguish in most cases the original sex of the embryo by a gross examination of the gonads. With low dosages of female hormones there are also certain recognizable differences in the oviducts of the females and transformed males. In the former the oviducts are normal, whereas in the latter they appear as discontinuous remnants at different levels on both sides of the body. With higher dosages the oviducts of both sexes become anatomically similar and even indistinguishable.

In contrast to the males, where the gonads are strongly modified in the female direction, the zygotic females fail to show any such intersexual modifications. The chief effect in the female is upon the oviducts. There is little or no change in the size and form of the ovaries (Figs. 7 and 8). Histologically the left ovary is typical except for an apparent sloughing-off of part of the cortex in three embryos and a distinct reduction of cortex in one case (E 15–14). The right ovary is structurally normal, however; in three cases several lumps of cortex occur on the surface. These differ in two cases from those occasionally present in the normal right ovary by being somewhat thickened.

Several grades of effects are seen upon the oviducts. With a low dosage (0.05 mg., 0.2 mg., and 0.4 mg. of theelin or theelol) they are either typical (forty-four cases) or the right oviduct is from two to three times as long as in the normal females (seventeen cases); in one of these cases in which the right oviduct has increased in length, the shell gland is missing in the left; in three cases (0.05 mg. of theelin or theelol) the right oviduct, although normal in length, has a swollen portion resembling a shell gland. With higher dosages (1.0 or 2.0 mg. of theelin or theelol) the right oviduct is not only increased in length but is tremendously swollen, and the left oviduct is likewise much swollen. Both oviducts usually exhibit alternating swollen and non-swollen portions, such as may be seen in Figures 7 and 8. A histological examination reveals that the inner epithelial wall of the oviduct is much folded upon itself.

EFFECTS OF MALE-HORMONE PREPARATIONS

Bull-testis tissue and male human urine were the sources of the male hormones used in these experiments. Data concerning the dosage given, the solvent used, the total weight of hormone preparation injected, and the percentage of embryos surviving are given in Table II. As many as eleven different preparations from urine were tested. These vary in hormone content from 7.9 to 150 S.B.U. (standard bird units), and in some cases it will be seen that some female hormone (6.3 I.U. [international units]) is present or its equivalent of crystalline theelin has been added as in E 9. Three preparations from bull's testis were tested. The doses were 10 and 200 S.B.U. of male hormone; female hormone was absent in all testis tissue preparations used. The standard bird unit used by us at the time of these investigations is the equivalent of 1.5 I.U. of male hormone.

A further examination of the table will show a considerable range in percentage of surviving embryos among the different preparations. It is apparent that some are much more toxic than others, regardless of the hormone content. Even such "highly purified bull-testis preparations" as E 22 and E 23 were apparently quite toxic. Undoubtedly, the physiological responses and the greater toxicity are in part due to the impurities found in the urine and testis-tissue products (cf. survival frequency in Tables I and II).

A total of 114 embryos has been examined macroscopically for modifications resulting from the injection of the various male hormone preparations. Of these, 82 received the various urinary extracts and 32 the bull-testis extracts. The gonads, and in some cases the oviducts and Wolffian ducts, of 36 (19♀ : 17♂) of the former and 17 (3♀ : 14♂) of the latter were histologically studied. In the case of the bull-testis extract, a greater number of male gonads was selected for histological examination in order to assure ourselves that no traces of ovarian cortex were present which might not have shown up macroscopically. Unlike the urinary products, this extract fails to bring about the development of an ovotestis.

Action of urinary hormone preparations upon male embryos.—In the majority of males the right testis is usually normal in size, shape, and histology; the left testis in one-third of them is likewise normal and in two-thirds of the cases has more or less the structure of an ovotestis. The latter, in some cases at least, resemble in structure and form the ovotestis of the embryos treated with theelin and theelol. The oviducts are either absent or more or less rudimentary. In all cases the Wolffian ducts have undergone hypertrophy.

As noted above for embryos injected with theelin and theelol, the most reliable key to the degree of intersexuality attained is the structure of the left testis. Consequently,

TABLE II

SUMMARY OF DATA ON EFFECTS OF MALE SEX HORMONES

Source	Hormone*		Dosage		No. of Eggs Injected	Survival on 10th–20th Day	Percentage of Survival	Comments
	Prep. No.	Milligrams Injected	S.B.U.†	I.U.‡				
Male human urine	E 1	5	9.6	6.3	47	17	36	
	E 2	3.3	10	0	51	16	31	
	E 3	2.4	10	0	37	20	54	
	E 5	11.8	15.8	0	34	1	3	
	E 6	5.9	7.9	0	24	1	4	
	E 7	54	12	?		0		
	E 8	?	0	6.3	40	3	7.5	Surviving embryos small and abnormal
	E 9	5	9.6	6.3	35	23	66	6.3 I.U. theelin added
	E 19	25	100	0	24	1	4	
	E 20	36	150	?	26	0	0	Same as E 3 except concentration
	E 21	9	50	?	12	0	0	
Bull testis	E 10	0.8	10	0	36	31	86	
	E 22	10	200	0	14	0	0	
	E 23	35	200	0	12	1	8	

* Dissolved in 0.1 cc. ethylene glycol.

† S.B.U. = Standard bird unit of male hormone. ‡ I.U. = International units of estrogenic activity.

the seventeen male embryos selected for histological study are grouped according to the grade of transformation of the left testis as follows: (1) normal; (2) rudimentary ovarian cortex present; (3) ovarian cortex definite, medulla scanty; (4) both ovarian cortex and medulla increased; and (5) ovarian cortex and medulla predominant, few testicular cords remaining.

1. *Normal.*—In five embryos, one injected with preparation E 1 and four with E 3, both testes are found to be normal in shape and histology. In two cases (E 3) a few testicular cords connect with the free surface of both testes. In all cases the oviducts are missing, as in normal males, and the Wolffian ducts exhibit various degrees of hypertrophy, ranging from a diameter slightly greater to as much as six times the normal.

2. *Rudimentary ovarian cortex.*—Two embryos, respectively injected with prepara-

tions E 2 and E 3, are in every respect typically like those described above, except for small amounts of persisting incipient cortex on the surface of the left testis. Structurally it is an epithelial layer containing germ cells, which is occasionally thickened, giving the appearance of abortive attempts to form a definite cortex. Testicular cords connect with the surface in both the right and left testes of both embryos. The right testes appear normal.

3. *Ovarian cortex definite; medulla scanty* (*ovotestis*).—Five embryos, one injected with E 1, one with E 2, two with E 3, and one with E 9, exhibit this grade of transformation. The right testis is normal structurally; but the left, although predominantly made up of testicular tissue, has two distinct ovarian components, cortex and medulla. The amount of cortex is quite variable. It may be either a thin or thick layer covering the entire outer surface of the testis or a thick layer covering the posterior half of the testis (two embryos). In the latter cases the gonad is anteriorly a testis, whereas posteriorly it is an ovotestis. The posterior portion is flattened like an ovary. See Figure 10. In all cases a few distinct medullary cords occur between the cortex and testicular tissue. The right testes in all five embryos appear normal.

Oviducts are absent in two of the embryos showing this grade of intersexual condition; in two they are persisting on each side as anterior portions along the side of the Wolffian bodies; and in one case there is an anterior trace of oviduct on the right side only (Fig. 10). The degree of hypertrophy of the Wolffian ducts is approximately the same as noted above, varying from two to eight times the normal diameter (Fig. 10).

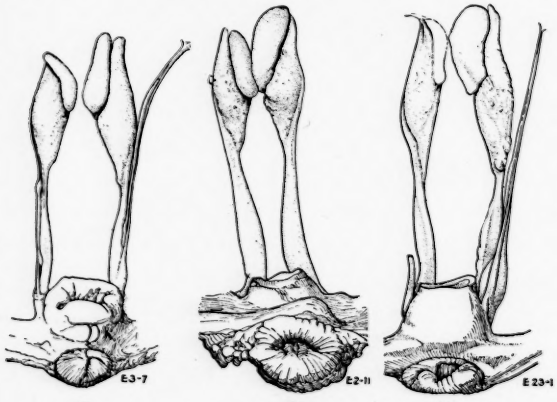
4. *Cortex and medulla increased* (*ovotestis*).—Four embryos (one each injected with E 1 and E 2, and two with E 3) exhibit a higher grade of sex-transformation than those just described. The chief advances noted are (1) a definite increase in medullary cords, forming a distinct layer in some cases, (2) the occurrence of a few medullary cords in the right testis (a few lumps of ovarian cortex occur on the right testis in three cases), and (3) the persistence of oviducts usually on both sides as short rudimentary portions situated anteriorly or at the middle. In one embryo (E 3–7) the left oviduct is complete and part of it has a lumen (see Fig. 9). The Wolffian ducts exhibit no great differences with respect to degree of swelling from those previously described.

As noted for the preceding grade of reversal, the ovarian cortex exhibits some variations in thickness and distribution. In general it is thicker over the posterior half of the testis.

5. *Ovarian cortex and medulla predominant; a few testicular cords.*—One embryo receiving urinary preparation E 9 exhibits the most extreme degree of sex-reversal encountered in our study of the effects of urinary extracts (see Fig. 20). In this case the left gonad resembles closely a normal left ovary in shape and structure. The thickness of the cortex and amount of medulla are quite similar to that found in the normal. With the exception of a few testicular cords situated at the hilus, the testis has the appearance of a left ovary. The right testis has likewise undergone some modification in the female direction, as is shown by the appearance of a few distended medullary cords among the testicular cords, which greatly predominate in number. On the surface of the right testis a few nodules of typical ovarian cortex are found. Both oviducts are present. The left is complete, terminating anteriorly in an ostium; it consists of tubular and threadlike portions. The right is tubular and extends anteriorly as far as the level of the right testis. The Wolffian ducts show the same grade of hypertrophy as was noted for all of the previously described cases.

Action of urinary extracts upon female embryos.—Two types of modification of the sex organs and ducts are seen among the nineteen female embryos examined histologically. Four embryos, one each receiving preparations E 1, E 3, E 5, and E 9, exhibit the first type of modification. The Wolffian ducts are hypertrophied to the same extent as in the male embryos described above. The ovaries and oviducts are typical in form and structure (Fig. 12) except for the appearance of lumps of ovarian cortex on the right ovary in three cases. In two cases (E 3–6 and E 9–3) the cortex on the left ovary appears thinner than normal, being absent altogether in places.

The remaining fifteen embryos, injected with preparations E 1, E 2, E 3, and E 9, present modifications of the second type. The hypertrophy of the Wolffian ducts in



9 10 11

FIG. 9.—Reproductive system of a genetic male embryo (E 3–7) injected with a male-urine extract containing 10 S.B.U. of ♂ sex hormone. Posterior half of left gonad (ovotestis) ovary-like; right a normal testis. Both oviducts persist; Wolffian ducts hypertrophied. ×2.5.

FIG. 10.—Reproductive system of a genetic male embryo (E 2–11) injected with a male-urine extract containing 10 S.B.U. of ♂ sex hormone. Left gonad (ovotestis) flattened like an ovary; right a normal testis. Wolffian ducts hypertrophied. Oviducts missing except for trace seen on outer surface of right Wolffian body. ×2.5.

FIG. 11.—Reproductive system of a genetic female embryo (E 23–1) injected with bull-testis extract containing 200 S.B.U. of ♂ sex hormone. Normal ♀ except for hypertrophy of Wolffian ducts. ×2.5.

ten embryos has reached an extreme degree (from ten to nineteen times their natural size). This effect is illustrated in Figure 14. By far the most striking effect, however, is seen upon the oviducts. They are either missing (Fig. 14) or more or less rudimentary on one or both sides (Fig. 13).

The ovaries present the same asymmetrical differences in size, form, and structure as the normal. Nodules of cortical tissue, however, appear more frequently (ten cases) on the surface of the right ovary than is characteristic of the normal. In one left ovary (E 3–12) the cortex is definitely thinner than normal. In three of the left ovaries (E 2–14, E 2–7, E 2–18) cords appear occasionally in the medulla that slightly resemble testicular cords.

Action of bull-testis extracts.—Male hormones from this source, in contrast to the urinary extracts, have very little action upon the sexual apparatus. A histological examination of fourteen of the sixteen males obtained shows that, as a rule, the testes are normal in form and structure. In eight of these, testicular cords in the left testis connect with the surface. In five cases where this connection occurs, dark-staining cells appear to be proliferating from the cord. In two embryos (E 10–17, E 10–8) traces of persisting incipient cortex (in the form of an epithelium containing some germ cells) are found. The oviducts are completely absent in all cases except one, E 10–17, where a trace of an anterior portion of the left occurs. Whether this is to be causally connected with the presence of ovarian cortex on the left testis just noted is problematical. The Wolffian ducts are typical in size and form in all embryos with the exception of three, where they are very slightly swollen.

Since a careful macroscopic examination of the fifteen females receiving 10 S.B.U. revealed no abnormalities either of gonads or ducts, only two were selected for a histological study. These, according to expectation, gave a perfectly normal picture. In both, traces of ovarian cortex in the form of small lumps were found on the right ovaries. The one surviving embryo, a female from the series receiving 200 S.B.U., exhibiting a distinct hypertrophy of Wolffian ducts, was examined histologically (see Fig. 11). The left ovary, although typical in its general form and structure, is characterized by a conspicuous absence of germ cells (cortex) over a relatively large area anteriorly. The right ovary of this same individual, like the aforementioned females, shows a trace of cortical tissue.

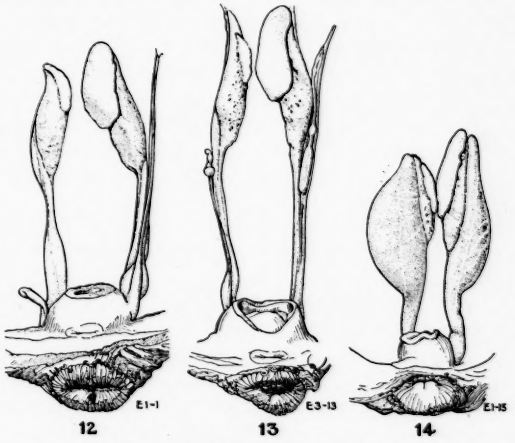


FIG. 12.—Urinogenital system of a genetic female embryo (E 1–1) injected with male-urine extract containing 9.6 S.B.U. of ♂ hormone and 6.3 I.U. of ♀ sex hormone. Normal ♀ except for hypertrophy of Wolffian ducts. ×2.5.

FIG. 13.—Urinogenital system of a genetic female embryo (E 3–13) injected with male-urine extract containing 10 S.B.U. of ♂ hormone. Ovaries normal; oviducts rudimentary; Wolffian ducts hypertrophied. ×2.5.

FIG. 14.—Urinogenital system of a genetic female embryo (E 1–15) treated with male-urine extract containing 9.6 S.B.U. of ♂ hormone and 6.3 I.U. of ♀ sex hormone. Ovaries normal; oviducts entirely absent; Wolffian ducts extremely hypertrophied. ×2.5.

DISCUSSION

Asymmetrical differences in response of the testes.—One of the outstanding results of this investigation of the action of sex hormones is the development in the zygotically determined male embryos of an intersexual composition of the left gonad while the right gonad usually remains a normal testis. The differences in response of the right and left gonads to the hormones is attributed to differences occurring in the normal development of the testis rudiments on the two sides of the body. The left testis passes through a stage

extending from at least the seventh to the eleventh day, during which a definite germinal epithelium containing germ cells is present over the entire free surface. The right testis, on the other hand, usually shows no such structure, although occasionally scattered traces of it may be found on its surface. It is the germinal epithelium of the left testis which becomes activated by the female hormones and develops into ovarian cortex. Thus the transitory germinal epithelium is shown to be an incipient ovarian cortex. On the basis of the structural foundations of the right testis a low frequency in development and a scattered distribution of ovarian cortex is expected. The occasional finding (five cases) of scattered nodules of ovarian cortex on the right testis following injection of female hormones is in perfect harmony with this expectation.

It is evident from these considerations, therefore, that the left testis rudiment is potentially bisexual, whereas the right testis is usually unisexual. Thus, for the first time the embryonic testes have been demonstrated to possess the same fundamental asymmetrical difference in bilateral organization that has long since been shown to be characteristic of the left and right ovaries of the domestic fowl. It is of especial interest in this connection to point out that as early as 1886 Laulainé interpreted the germinal epithelium of the developing left testis, found present between the seventh and twelfth days of incubation, as a "couche ovigène." He regarded this layer as a homologue of the ovarian cortex, since the time of its appearance coincides with the beginning of cortical proliferation in the ovary, i.e., immediately following the initiation of sex-differentiation. Our experiments demonstrate that this structural homologue has the potencies of an ovarian cortex.

Degree of intersexuality attained in males.—The degree of sex-reversal attained in the male embryos is roughly proportional to the quantity of female hormone injected. With the lowest dosage given (i.e., 0.05 mg. theelin or theelol) the left gonad remains primarily a testis, the ovarian tissues varying from none to a definite cortex and medulla; and the oviducts are missing. With intermediate dosages of 0.2 and 0.4 mg. of theelin or theelol both ovarian cortex and medulla are characteristic components, and the amount of testicular tissue is reduced. The right testis in some cases begins to show medullary tissue, and oviducts tend to persist. With the highest dosages (1.0 and 2.0 mg.) ovarian cortex and medulla predominate, the testicular tissue being reduced to a few cords; the right testis exhibits a higher frequency of transformed sex cords; and the oviducts (either partly or completely) invariably persist and are hypertrophied. Lastly, in two embryos, one receiving 2.0 mg. of theelin, the other 1.0 mg. of theelol, the left testis is completely transformed into a left ovary and the right testis largely into a right ovary; the oviducts are strikingly similar to those of a normal female. By varying the quantity of folliculine given, Wolff and Ginglinger (1935*b*) have obtained similar grades of intersexuality.

Although a definite relationship is thus shown between dosage and degree of effect, there is considerable range in the effect produced with each dosage of hormone given. For example, an embryo receiving a dosage of 0.4 mg. of theelin may show an effect as great as that usually produced by 1 mg. of theelin or theelol; or one receiving 0.05 mg. of theelin or theelol may be nearly as much modified as one receiving 0.2 or 0.4 mg. of theelin or theelol. Although no definite evidence is at hand to explain such variations in response to a given concentration, it is suggested that the time that the hormones begin to act during the early stages of sex-differentiation is at least one factor. This may depend upon a delay in the time after injection that the hormones become available for absorption from the egg albumen or upon variations in their rate of absorption in the

different embryos. Their availability may depend upon the locus of injection, which is more or less variable, although attempts were made to keep it relatively constant. Also, it is probable that some of the female hormone may have been precipitated with the egg albumen. Another possible interpretation is that the quantitative effect of the hormone varies in accordance with the condition of balance of the zygotic sex-determining factors in the different individuals.

It becomes clear from the foregoing discussion that we are not dealing with an all-or-none principle in the modification of the male embryos, since low dosage fails to do much more than superimpose ovarian cortex upon a testis while high dosages are required to bring about an approximation of complete reversal of the sex of the gonad and a retention of the oviducts. That is, a rather complete range of intergrades between a testis and an ovary is found. This result is in rather sharp contrast to the all-or-none type of reaction found by Lillie (1923, pp. 60 and 70) to be characteristic in the formation of the freemartin. The evidence for this conclusion came from the observation that the freemartins "form an exceedingly well-defined group without intergrades to normal female or normal male;" and, furthermore, that there is no apparent correlation between the size of the vascular anastomosis and the degree of intersexuality (p. 60).

An interesting question presents itself next as to the minimum dosage necessary to activate the incipient cortex. Although no special study of this has been made, the data are suggestive. Since 0.05 mg. of theelin or theelol bring about slight or no effects, this appears to approximate the concentration of threshold effects under the conditions of the experiment. Breneman (1935) used lower dosages but injected into the air chamber, and reports a change in form of the gonads (increase in female type of gonads) but without formation of cortex. Such a change in the form of the gonad is somewhat puzzling, since in our experiments a definite correlation occurs between the assumption of the flattened form and the formation of ovarian cortex on the left testis. According to Wolff and Ginglinger (1935*a*), a dose of from 35 to 50 I.U. of folliculine dropped upon the chorion prior to the eighth day may bring about an intersexual condition in male embryos.[3]

Manner of action of the female hormones.—The primary step in the development of intersexuality in the males is apparently the activation of the incipient ovarian cortex by the female hormones. This is largely deduced from the fact that the lowest dosage used initiates, as a rule, only the formation of ovarian cortex. Whether this activation results from the direct action of the hormones or indirectly is not clear. Assuming the correctness of the general principle that genes act locally, whereas hormones are effective on parts remote from the place of their origin, it seems probable that the hormones do not act directly upon the cortical rudiment of the testis but in some indirect way. That they act through the hypophyseal mechanism or some other chain of processes is indicated. A preliminary histological examination of the hypophyses of the injected embryos, although they were not suitably fixed for cytological study, reveals some abnormal features. Also, it is conceivable that the female hormones have an inhibitory action upon the development of the male sex cords, whereupon the latent potencies of the cortical rudiment express themselves.

Although the activation of the incipient cortex appears to be the first step in the process of reversal, it is not known whether other events are causally related to this. A ques-

[3] Since writing the foregoing, we have found that 0.035 mg. of theelin dissolved in propylene glycol, dropped upon the chorion of 5-day embryos, brings about in males the development of an ovotestis equivalent to that produced by much higher dosages when the injection is made into the albumen.

tion of considerable interest is whether the transformation of the testicular cords into medullary cords is the result of the presence of ovarian cortex or of the positive action of the injected female hormones. It is known from the studies of ovariotomy in the fowl after hatching that ovarian cortex has an inhibitory influence upon ovarian medulla; so in these experiments the possibility must be admitted that the testicular cords may assume the form of medullary cords under the influence of the superimposed cortex. It is important to point out in this connection that the order of transformation of the testicular cords into ovarian medulla is from the cortex inward toward the hilus, the cords here retaining their male form last. Also, that the increase of medullary tissue is roughly proportional to the amount of ovarian cortex formed. This appears to be true for gonads of embryos injected with the lower dosages of hormone. However, with the highest doses (1 mg. or over), although there is no apparent increase in the thickness of the cortex over a maximum thickness brought about by a lower dose (such as 0.4 mg.), more testicular cords are replaced by medullary tissue. This suggests that the injected hormones act directly, or in conjunction with the cortex, upon the testicular cords in bringing about their transformation.

It is also probable that other parts of the reproductive system are correlated by similar or other processes with each other. The possibility must also be admitted that the persistence and hypertrophy of the oviducts may be correlated with cortex development or result from the direct action of the injected hormones. That the hormones act directly upon the oviducts is indicated, since (1) oviducts often fail to persist although ovarian cortex is well developed, and (2) they invariably persist and, as a rule, are well developed in embryos receiving the highest doses of hormone. Finally, there is the question as to whether variations in persistence and degree of development of oviducts are to be correlated with laterality or anteroposterior level. Owing to fundamental asymmetrical differences in the development of the oviducts in normal females, it might be expected that the left oviduct in the feminized males would be more likely to persist or be better developed than the right. In the highly modified males the left oviduct is found to approximate closely the normal in form and structure, while the right is less well-developed but always longer and better developed than the right of a normal female. In males showing a low grade of transformation, short portions of the oviducts tend to persist with equal frequency on both sides of the body. Furthermore, they tend to appear first as short portions at the anterior end, next as short portions lateral to the cloaca, and lastly a mid-portion is added completing the oviduct.

On the basis of these findings it is evident that sex hormones do not originate any morphological component but merely activate or alter the development of those already laid down in normal development. Each female structure developed in the male may be readily accounted for. The ovarian cortex arises from an incipient cortex, a normal constituent of the embryonic left testis. The ovarian medulla results from a transformation of testicular cords into their homologues, the medullary cords; and the oviducts are normally developed at a certain stage in every male. In the formation of ovarian cortex on the testis and the persistence of the oviducts of the chick we have an example of activation by female hormones. This is in contrast to an apparent suppression of cortex by male hormones in the freemartin gonad (Lillie, 1917). There is also some evidence that the male hormones have an inhibitory effect upon the cortex of the left ovary of the chick, since it occasionally shows defects in cortical development. The transformation of testis into medullary tissue probably has the nature of a suppressing effect, since in the

absence (surgical removal in post-hatched chicks) of cortex the primary sex cords assume the male form. The hypertrophy of the Wolffian ducts, normally present in both sexes, is an example of a stimulative effect.

Sex hormones and the genic mechanism of sex-differentiation.—Granting that in the chick the male zygote contains the genetic factors for both sexes, it follows that the primary determining constitution of the male embryo is the result of a predominance of male over female factors. We may, therefore, regard the intersexual condition of the left gonad as due to an intensification of the female factors of a male zygote by the female sex hormones. The intensity of the female-determining effect appears, as we have seen, to depend upon quantitative variations of the hormone injected. A graded series of structural changes between a male and a female is seen. In such anatomical females the original male producers have become completely overbalanced by the female sex hormones. In the intersexes the balance between the opposing male (genic) and female (hormonic) producers is somewhat variable, the grade of development of femaleness or maleness being easily controlled by the quantity of female hormone administered.

A final question of much theoretical significance is whether the female hormones partake of the nature of the influence which directly emanates from the genes in bringing about sex-differentiation. Although no answer can be given, it is important to point out the striking resemblance between the action of genes and of hormones in determining the direction of differentiation of sex characters; viz., both have been demonstrated as effective agents in influencing the process of sex-expression in the embryo.

Effective period of hormone action.—In the formation of the freemartin, Lillie (1917) has emphasized the importance of the time of onset of hormone action. Vascular connections are suitable for the introduction of male hormones from the male twin into the circulation of the female twin at or just before the beginning of morphological sex-differentiation. Although in the chick the hormones were injected into the albumen prior to the time of origin of the gonad and sex-duct rudiments, no direct evidence is available as to just when they enter the circulation of the embryo. It seems certain, however, that this occurs prior to the onset of sex-differentiation, which begins between the sixth and seventh day of incubation. Certainly a period of 5 days following the day of injection is ample time for the absorption of a considerable amount of the hormone from the albumen.

As was pointed out in a previous paper (Willier, Gallagher, and Koch, 1935*c*), the effective period for obtaining a response to the action of the female sex hormones in the chick probably extends from about the time that sex-differentiation begins until the retrogression of the incipient cortex and oviducts sets in, or at most when this process is complete. A study of normal development reveals that retrograde changes begin to occur about the ninth day and are completed at approximately the eleventh day. By injecting folliculine (dosage not stated) into the allantoic sac, Dantschakoff (1935) has ascertained that differences in sensitivity of the gonad to the hormone at various stages in development do occur. She found that, if the injections were made into 10-day embryos, the effect was slight; intermediate in the case of 6-, 7-, and 8-day embryos; and that injections into 4-day embryos brought about the greatest degree of sex-reversal. Although these results indicate that the most effective period definitely precedes sex-differentiation, the effectiveness gradually fades off as retrogression of the incipient ovarian cortex and of the oviducts proceeds. That it disappears altogether when the processes of retrogression are completed seems very probable. Indeed, Wolff and Ginglinger

(1935*a*) have found that if injections of folliculine are made into 12-day embryos no intersexuality of the gonad results.

Specificity of action of hormones upon sex ducts.—The data show quite clearly that male and female sex hormones stimulate, respectively, potential male (Wolffian) and female (Müllerian) sex ducts. Neither male nor female sex hormone has affected in a single instance the heterologous sex duct. It is the specific nature or potency of the sex duct, irrespective of the zygotic sex-constitution of the embryo, which determines whether or not it responds.

Male hormone from human urine appears to have an inhibiting action upon oviducts of genetic females. This is brought out by the rudimentary character of the oviducts, especially of the left, or by their complete disappearance. Such an effect appears to be the result of the direct action of the hormone injected, since the ovaries of the same female are apparently unmodified. This significant finding may indicate that in the normal male the oviducts undergo retrogression under the influence of male hormones produced by the differentiating testis.

No inhibiting action of female hormones upon Wolffian ducts has been discerned. Such an effect, although theoretically expected, is less easily detected since the Wolffian ducts persist in the female in a non-functional state even after hatching.

Peculiarities in action of the male-hormone preparations.—The male-urine and bull-testis preparations used in these studies exhibit different qualitative and quantitative effects on the reproductive organs and ducts of the chick embryo. This is not entirely unexpected, in view of recent chemical and biological studies. The chemical differences in the testis-tissue hormone and the urinary male hormone were first shown by Gallagher and Koch (1934). Biological differences in the concentrates from the two sources were clearly shown by the comparative studies on capons and rats by Dingemanse, Freud, and Laqueur (1935), Laqueur *et al* (1931), and also by similar studies on the pure crystalline products by Butenandt and his associate (1931, 1934) and by David, Dingemanse, Freud, and Laqueur (1935). The subsequent confirmation of these chemical and biological observations has resulted from the synthetic degradation studies on cholesterol by Ruzicka and his co-workers (1934, 1935) and by Butenandt *et al* (1934, 1935), and by the biological studies of Korenchevsky and his collaborators (1932, 1935), of Parkes (1935), of Callow and Deanesly (1935), and of Tschopp (1935). These studies show that urinary preparations contain androsterone and dehydroandrosterone, whereas the testis-tissue preparations yield testosterone. By the capon comb-growth test, testosterone is found to be six to ten times as active per unit weight as androsterone, and dehydroandrosterone is only about one-half as active as androsterone. In the castrated male rat, the relative potencies of these substances in the regeneration of the seminal vesicles and prostate are still greater. By this test, testosterone is found to be approximately twenty-five times as potent per unit weight as androsterone, and dehydroandrosterone is practically without action.

Both preparations have a similar qualitative effect, i.e., that of hypertrophy, upon the Wolffian ducts (potential vasa deferentia) in both sexes. This may be interpreted as a true male-hormone action brought about by testosterone in the bull-testis concentrates and by androsterone in the urinary preparations. However, when equal dosages, as determined by the comb test, are given, the latter brings about a greater hypertrophy of the Wolffian ducts than the former. This quantitative difference in the response of the

ducts is rather surprising, since the order of effectiveness of the hormones is the reverse of what is expected, assuming that their diffusion in the eggs has been uniform. In the foregoing paragraph it was pointed out that the relative potencies of these preparations and of the pure substances isolated therefrom show quantitative differences in their action upon the capon comb growth. This difference is still more striking when their action is tested on male accessory reproductive organs. Per capon unit, the testis-tissue hormone is from three to five times more active on the seminal vesicles and prostate of the rat than androsterone or the urinary product. In the chick embryo, however, the hypertrophy of the Wolffian ducts is greater per capon unit of urinary product than of testis-tissue preparation.

A striking qualitative difference is also seen in the action of the male-hormone preparations upon the gonad rudiment of the male embryo. The male-urine preparation activates the incipient ovarian cortex of the testis, whereas the bull testis does not. The former, regardless of whether the rat vaginal smear shows the presence or absence of female sex hormone, brings about the same development of ovarian cortex on the left testis as does theelin and theelol, although in general to a lesser degree. It is apparent, therefore, that the male-urine preparation contains a substance which is similar in its physiological action to that of theelin and theelol.

It is now well known that when urinary male-hormone preparations are freed from estrogenic female-hormone activity of the theelin type, androsterone and dehydrandrosterone are still retained in the male-hormone fraction. The studies of Butenandt and his associates (1935) and of Deanesly and Parkes (1936) have shown that these two substances possess weak theelol-like actions on the immature female rat. In view of these findings we may account for the activation of the incipient cortex by the theelol-like action of the urinary preparations. Still another factor may be involved in the action of the urinary preparations, as is suggested by the findings of Dorfman, Gallagher, and Koch (1935). They give evidence that, after removal of the alkali-soluble theelin and theelol from urinary preparations, still another substance is retained which enhances the action of theelin on the vagina and uterus. Although testosterone has a very low theelol-like activity, it is apparently not sufficiently great to activate the incipient ovarian cortex of the male gonad primordium.

It is obvious that a satisfactory interpretation of the findings with male-hormone preparations is impossible at the present time and that studies with various pure male hormones in crystalline form and of known structure should simplify the problem. Studies with these pure products may eliminate the toxic effects noted in some of our studies and consequently enable us to inject higher doses. These studies are now in progress.

SUMMARY

1. This report deals with the effects upon the development of the sex glands and ducts resulting from injections of male and female sex-hormone solutions into the hen's egg at a stage prior to the beginning of sex-differentiation.

Eggs from single-comb Brown Leghorns and from a cross, the embryos of which are distinguishable as to sex by sex-linked plumage, were used.

2. Various concentrations of theelin, theelol, bull-testis, and male human urine preparations, as well as the solvent used, were tested.

3. A single injection of 0.1 cc. sex-hormone solution (in ethylene glycol) of varying

potency was made into the albumen of eggs of 48 hours' incubation, and development continued until the nineteenth day.

Over 350 embryos, including both experimental and normal controls, have been examined.

4. Upon the injection of the female hormones, theelin and theelol, the right and left ovaries of genetic females as a rule remain unaffected. The left testis of the genetic males is, however, markedly altered in form and structure, appearing most often as a flattened ovary-like body consisting of both ovarian and testicular tissues (ovotestis). In two extreme cases (1.0 and 2.0 mg. dosages) it is practically indistinguishable from a normal left ovary; i.e., reversal is complete. The right testis remains unchanged except in those cases exhibiting complete or nearly complete reversal of the left testis, whereupon it appears reduced in size, flattened, and contains ovarian medullary tissue; its testicular character is never entirely lost.

Oviducts in the males persist partially or completely. In the females the length of right oviduct is increased. With high dosages (1 mg. or over) the oviducts of both sexes become hypertrophied. Wolffian ducts in both males and females appear unmodified.

The degree of sex-reversal attained is roughly proportional to the quantity of female hormone injected.

5. Bull-testis preparations produce no observable effect on the gonads or oviducts of either sex; the Wolffian ducts may undergo slight hypertrophy.

Male-urine preparations, while ineffective in producing changes in the gonads of the females, bring about similar grades of transformation of the left testis into an ovotestis, as did the female sex hormones; the right testis likewise undergoes some modification in the female direction.

Oviducts are absent in the males and rudimentary or absent in the females. The Wolffian ducts of both sexes are consistently and tremendously swollen.

6. The asymmetrical difference in response of the testes to female hormones (and to certain male-urine preparations) is attributed to an incipient ovarian cortex occurring on the left testis at an early stage in its development, whereas the right testis usually lacks such a structure.

The left testis rudiment is potentially bisexual; the right, unisexual.

7. Activation of the incipient ovarian cortex appears to be the first step in the process of reversal. Whether the transformation of the testicular cords into medullary cords and the persistence and hypertrophy of the oviducts is the result of the mere presence of ovarian cortex or of the positive action of female hormones is somewhat problematical.

8. A specificity of action of the hormones on the sex ducts is shown, since male and female hormones stimulate, respectively, potential male (Wolffian) and female (Müllerian) sex ducts, regardless of the zygotic sex-constitution of the embryo.

Male hormone from human urine appears to have an inhibitory action upon oviducts of genetic females, since they are rudimentary or absent altogether.

9. Biological differences in the action of the male-urine and bull-testis preparations upon the sex glands and ducts are to be correlated with chemical differences found therein.

The activation of the incipient ovarian cortex of the testis by the urinary preparation is ascribed tentatively to the presence of substances known to have a theelol-like action of a low grade.

ADDENDUM

After the foregoing paper was completed, Dantchakoff (*Bull. biologique*, **70**:241–307, 1936) and Wolff and Ginglinger (*Arch. d'Anat., d'Hist. et d'Embr.*, **20**:219–78, 1935) have published their results in full on the effects of folliculine. Although the method of administering the hormone to the embryo differs from ours, the results are in essential agreement. These authors, however, have failed to correlate the differential action of the female hormone upon the right and left testes with differences in their structural organization.

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PLATE I

FIGS. 15, 16, 17.—Photomicrographs showing ovotesticular structure of the left gonad from three genetic male embryos injected respectively with 0.2 mg. of either theelin (E 13–13) or theelol (E 15–7 and E 15–32). Note ovarian cortex, ovarian medulla (broad zone in Fig. 16), and testicular cords. Initial ×210.

PLATE II

FIG. 18.—Ovotestis (left gonad) of a genetic male embryo (E 25–3) injected with 1.0 mg. of theelin. Initial ×110.

FIG. 19.—Detail of a portion of ovotestis shown in Figure 18. Note arrangement of ovarian cortex, ovarian medulla, and testis cords in definite zones. Initial ×210.

FIG. 20.—Ovotesticular structure of left gonad of a genetic male embryo (E 9–10) treated with a male-urine preparation containing 9.6 S.B.U. of male hormone and 6.3 I.U. of female hormone. Initial ×210.

PLATE I

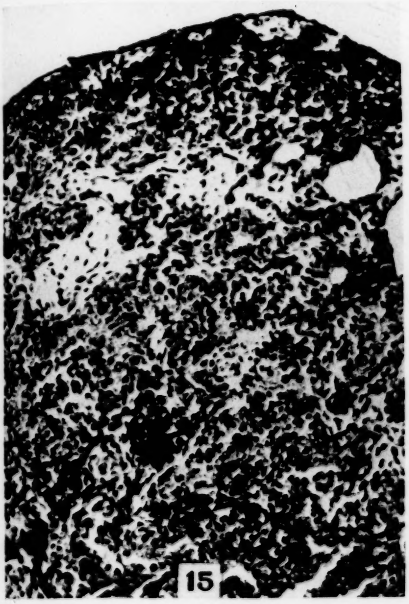

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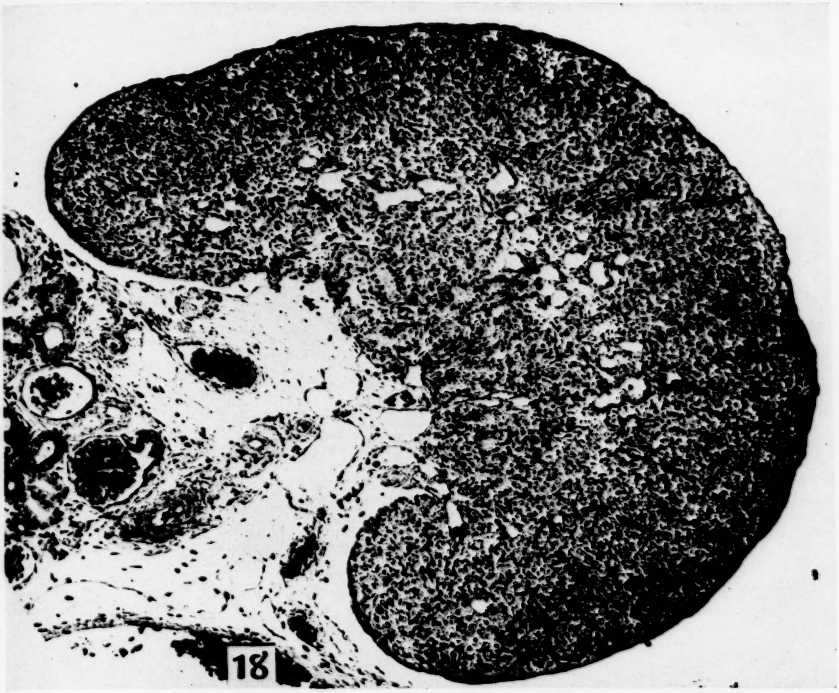
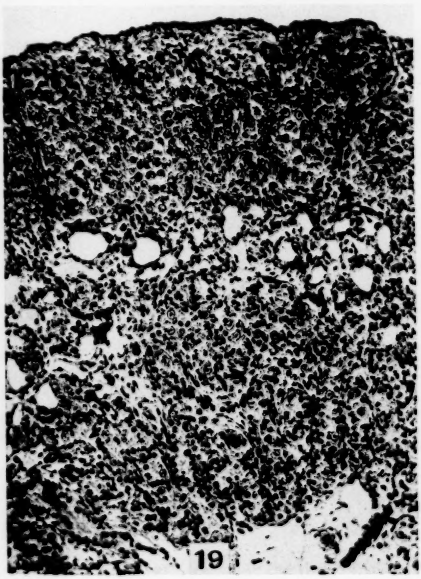
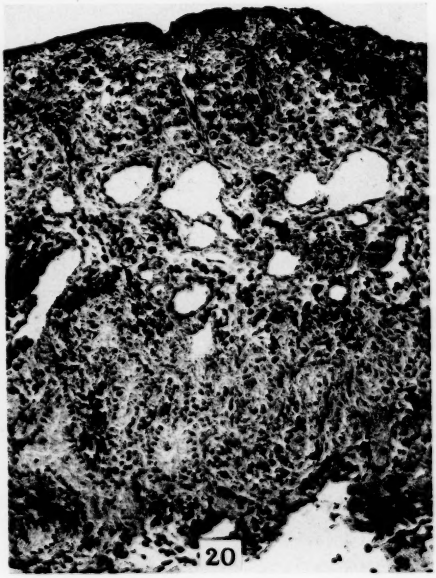
PLATE II







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